The background of the cover is a dark, textured grey. It features several faint, light-colored botanical illustrations. In the upper center, there is a detailed drawing of a plant stem with several long, narrow, pointed leaves and a cluster of small, round flowers at the top. To the right, there is another illustration of a plant with more complex, branching structures and small flowers. In the lower left, there are some smaller, less distinct drawings of plant parts, including what looks like a seed or fruit. The overall aesthetic is scientific and artistic.

Plant Pathogens and Their Biocontrol Agents

Diagnostics and Characterization

Edited by

S.J. Eapen, A. Kumar & M. Anandaraj

34

Plant Pathogens and Their Biocontrol Agents

Diagnostics and Characterization

Editors

S.J. Eapen, A. Kumar & M. Anandaraj



IIISR PUB - 35



INDIAN INSTITUTE OF SPICES RESEARCH
Calicut - 673 012, Kerala, India

Plant Pathogens and Their Biocontrol Agents: Diagnostics and Characterization

Published by

Director, Indian Institute of Spices Research, Calicut, Kerala - 673 012

Edited by

S.J. Eapen, A. Kumar & M. Anandaraj

Cover designed by

A. Sudhakaran

Printed at

Niseema Printers & Publishers, Kochi, Kerala - 682 018

ISBN : 81-86872-31-0

Copyright©2008

Indian Institute of Spices Research, Calicut, Kerala

All World Wide Web addresses are accurate to the best of our knowledge at the time of printing. The views expressed are those of the concerned authors. Neither the editors of this book nor Indian Institute of Spices Research assume any responsibility for any copyright violation or other proprietary right infringement.

Contents

Sl. No	Title	Authors	Page No.
	Foreword		i
	Preface		iii
	List of contributors		v
GENERAL			
1.	Biotechnological approaches for plant disease diagnostics and management	S Prakash Naik	3
2.	Bioinformatics packages for sequence analysis and practical applications	Dilip K Arora & Bandamaravuri Kishore Babu	33
METHODS AND PROTOCOLS			
3.	Isolation techniques for soil borne fungi, bacteria and nematodes	R Suseela Bhai & Santhosh J Eapen	45
4.	Preparation of reagents and DNA isolation	A Kumar, V Vinod	62
5.	Purification of plant viruses	A Ishwara Bhat	66
6.	Enzyme linked immunosorbent assay (ELISA)	A Ishwara Bhat	72
7.	Cloning of PCR product	A Ishwara Bhat	78
8.	RAPD PCR	T E Sheeja	84
9.	Amplified ribosomal DNA restriction analysis-ARDRA	A Kumar, Dinu Antony, V Vinod & R Aravind	90
10.	DNA microarrays in microbial studies	M K Rajesh	95
11.	Isoenzyme analysis	K S Krishnamurthy	103
12.	Primer designing	Santhosh J Eapen & S Balaji	106
13.	Construction of phylogenetic trees	Santhosh J Eapen & S Balaji	112
MOLECULAR CHARACTERIZATION			
14.	Biological characterization of plant pathogens	M N Venugopal	117
15.	Characterization of pathogens based on isozymes and allozymes markers	Shamina Azeez & B Chempakam	124
16.	Molecular approaches for studying the genetic diversity of fungal pathogens and biocontrol agents	M Anandaraj & A Kumar	134

17. Molecular approaches for analyzing the genetic diversity of <i>Phytophthora capsici</i> infesting black pepper	M Anandaraj, Sheji Chandran & A Kumar	137
18. Molecular characterization of bacteria	A Kumar, Dinu Antony, V Vinod & R Aravind	141
19. Molecular systematics- basic concepts	K Nirmal Babu & J Martin	146

DETECTION AND DIAGNOSIS

20. Role of diagnostics in disease management	M Anandaraj	155
21. Applications of genomics and proteomics in diagnostics	R Manimekalai	160
22. Serology and immunodiagnosis of plant pathogens	M Krishna Reddy	165
23. PCR based detection of plant viruses	A Ishwara Bhat	179
24. ITS based species identification – <i>Radopholus similis</i>	Dinu Antony & Santhosh J. Eapen	183
25. Applications of bioinformatics in diagnostics	Santhosh J. Eapen	186
26. IT based species identification	Santhosh J. Eapen	191
27. Plant quarantine – means and methods of detection and exclusion of plant pathogens	N Sathyanarayana & S Latha	196

BIOCONTROL

28. Isolation, identification and evaluation of fungal biocontrol agents	B Ramanujam	207
29. Exploitation of the antagonistic microbes for identifying novel antifungal genes and plant disease management	R Viswanathan & P Malathi	225
30. Microbial control of insect pests	S Devasahayam	252
Keyword Index		259



भारतीय
ICAR

भारतीय कृषि अनुसंधान परिषद

कृषि अनुसंधान भवन - 11

पूसा, नई दिल्ली 110 012

INDIAN COUNCIL OF AGRICULTURAL RESEARCH

KRISHI ANUSANDHAN BHAVAN-11

PUSA, NEW DELHI 110 012

डा. एच. पी. सिंह

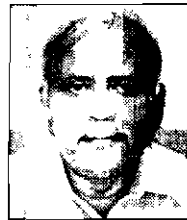
उप महानिदेशक

(बागवानी)

Dr. H. P. SINGH

Deputy Director General

(Horticulture)



FOREWORD

Biotic stress causes serious loss to the production of crops. Early diagnosis of the pathogen is essential to manage the biotic stress caused by pathogens. Since lack of accurate diagnosis leads to unacceptable, heavy crop losses and waste of precious resources, it demands for rapid and reliable techniques to detect the plant diseases well on time to adopt effective management strategies. There is an increasing use of molecular tools for genetic mapping of microorganisms to understand the pathogenic forms. This is used as decision support system for the management of diseases. New diagnostics and detection methods are being appreciated for the diagnosis, prevention, and management of plant diseases. Many of these developments have taken place mainly in a decade which has enhanced the capability for early and accurate diagnosis of biotic stresses.

In the above context, the three week ICAR sponsored training on 'Diagnostics and molecular characterization of pathogens of horticultural crops and their biocontrol organisms' was organized at IISR, Calicut, which had participation of scientists and teachers from different parts of the country. Based on the training a manual has been prepared which contains the information on the tools and technologies and is considered to be a useful compilation. I am sure this manual will serve as a useful tool for understanding the diagnostics. I compliment the editors for compiling this useful information together.

H. P. Singh
(H.P. Singh)

PREFACE

Biotechnology has given us new tools to improve food security and reduce poverty. Recent breakthroughs in biotechnology have led to rapid progress in our understanding of living organisms, both beneficial and pests of crops. Agriculture to be successful, a comprehensive knowledge of the fauna and flora is essential. A better understanding of microorganisms is made possible with increasing use of molecular tools. The three weeks' ICAR sponsored training on 'Diagnostics and Molecular Characterization of pathogens of Horticultural Crops and Their Biocontrol Organisms' was organized at Indian Institute of Spices Research, Calicut with this objective. This winter school was attended by twenty one participants selected from various ICAR institutes and agricultural universities. To give more impetus to the above theme, the lectures delivered during this training programme are compiled and brought out as an edited publication titled '*Plant Pathogens and Their Biocontrol Agents - Diagnostics and Characterization*'. By bringing out the training manual as a printed publication, the message is reaching out to a wider audience. We feel that this will be a handy resource to the plant protection students and scientists of the country who still rely on traditional methods to deal with these organisms.

This book is divided into five sections. The first section is a general one comprising of two chapters devoted to recent developments in Biotechnology and Bioinformatics in the perspective of a plant protection scientist. The next section comprises of chapters describing general methodologies and protocols useful for research on plant pathogens and biocontrol agents. Section III includes seven chapters pertaining to molecular characterization. Biological, biochemical and molecular approaches employed for characterization are discussed here. Various cutting edge techniques useful for detection and diagnostics are dealt with in Section IV. These include PCR based methods and applications based on serology, genomics and proteomics. There is an exclusive chapter on plant quarantine in this section. The last section is on biological control of plant diseases and insects.

We believe that there is a strong need for an introductory book on diagnostics and molecular characterization that provides detailed coverage of both the basic and applied aspects of biotechnology tools. This book is written primarily for crop protection scientists and students. We sincerely hope that the information contained in this volume must be of use for sharpening their skills. We are indebted to Dr Mangalarai, Director General, Dr. S. P. Tiwari, DDG (Edn.), ICAR, New Delhi for sanctioning the above training programme. We wish to thank Dr. H. P. Singh, honourable DDG (Hort.), ICAR, New Delhi for his keen interest in this programme and for writing the foreword for this publication. The efforts of all the resource persons who contributed chapters are thankfully acknowledged. We are deeply indebted to Director, IISR, Calicut for all the encouragement and support in this venture.

- Editors

LIST OF CONTRIBUTORS

Anandaraj M

Project Coordinator (Spices)
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Aravind R

Research Fellow
Division of Crop Protection
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Balaji S

Research Fellow
Bioinformatics Centre
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Bandamaravuri Kishore Babu

National Bureau of Agriculturally
Important Microorganisms
Mau, Nath Bhanjan, Uttar Pradesh
PIN - 275 101

Chempakam B

Head, Division of Crop Production
& Post Harvest Technology
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Devasahayam S

Head Division of Crop Protection
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Dilip K Arora

Director
National Bureau of Agriculturally
Important Microorganisms
Mau Nath Bhanjan, Uttar Pradesh
PIN - 275 101

Ishwara Bhat A

Senior Scientist
Division of Crop Protection
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Krishnamurthy K S

Senior Scientist
Division of Crop Protection
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Krishna Reddy M

Principal Scientist
Indian Institute Horticulture Research
Hessarghatta Lake Post
Bangalore, Karnataka
PIN - 560 08

Kumar A

Senior Scientist
Division of Crop Protection
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Latha S

Regional Plant Quarantine Station
Chennai, Tamil Nadu
PIN - 600 027

Malathi P

Senior Scientist
Sugarcane Breeding Institute
Coimbatore, Tamil Nadu
PIN - 641 007

Manimekalai R

Scientist Senior Scale
Central Plantation Crops Research
Institute, Kasaragod, Kerala
PIN - 671 124

Martin J
Research Fellow
Division of Crop Improvement and
Biotechnology
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Nirmala Babu K
Principal Scientist
Division of Crop Improvement
& Biotechnology
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Prakash Naik S
Project Coordinator
All India Coordinated Potato
Improvement Project
Central Potato Research Institute
Shimla, H.P.
PIN - 171 001

Rajesh M K
Scientist Senior Scale
Central Plantation Crops Research
Institute
Kasaragod, Kerala
PIN - 671 124

Ramanujam S
Principal Scientist
Project Directorate of Biological
Control
Bangalore, Karnataka
PIN - 560 024

Santhosh J Eap
Senior Scientist
Division of Crop Protection
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Sathyanarayana N
Deputy Director

Regional Plant Quarantine Station
Chennai, Tamil Nadu
PIN - 600 027

Shamina Azeez
Senior Scientist
Division of Crop Production
& Post Harvest Technology
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Shceja T E
Senior Scientist
Division of Crop Improvement
& Biotechnology
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Sheji Chandran
Research Fellow
Division of Crop Protection
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Suscela Bhai R
Senior Scientist
Division of Crop Protection
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Venugopal M N
Head, Cardamom Research Centre
Indian Institute of Spices Research
Appangala, Madikeri, Karnataka
PIN - 571 201

Vinod V
Research Fellow
Division of Crop Protection
Indian Institute of Spices Research
Calicut, Kerala
PIN - 673 012

Viswanathan R
Senior Scientist
Sugarcane Breeding Institute
Coimbatore, Tamil Nadu
PIN - 641 007

I

GENERAL

1

Biotechnological approaches for plant disease diagnostics and management

S Prakash Naik

Existence of human civilization is dependent on plants. Plants, like animals, are continuously exposed to various pathogens. It has been estimated that upto 40% of plant productivity in Africa and Asia, and about 20% in the developed world is lost to pests and pathogens. Approximately, one third of the losses are due to fungal, viral and bacterial pathogens, and the remainder is due to insects and nematodes. As evidenced by outbreaks of late blight of potato, victoria blight of oats and southern leaf blight of corn, the threats of serious epiphytotics still persist today. In addition to causing food shortages, diseased plants can also directly affect the health of human and livestock. One notable example is ergot poisoning resulting from toxins in the fruiting bodies of the fungus *Claviceps purpurea*, which can contaminate rye flour causing frightening syndrome typified by hallucinations, burning sensations, miscarriages, gangrene and in severe cases death. These concerns call for concerted efforts for diagnosis and eco-friendly management of important diseases in major food crops. Disease diagnostics has traveled a long way during last few decades starting from biodiagnostics to serodiagnostics, immunodiagnostics, monoclonal antibodies, recombinant antibodies, nucleodiagnosis, RT-PCR etc. However, large scale adoption of these sensitive techniques will depend upon development of affordable protocols in future.

Plants have evolved their own defense systems to combat against disease causing pathogens. During the course of a long-drawn coevolution of plants and pathogens, pathogens have developed means to circumvent defensive barriers elaborated by the plant to prevent infection for successful colonization on a particular host. Once such barriers are breached, the susceptible host plants are then subjected to selection pressures to develop counter measures that block invasion by the pathogens. These dynamic and ongoing evolutionary battles have resulted in the ramification of highly specific and extremely sophisticated attack strategies by the pathogens and equally elaborate defense responses by the host plants. Knowledge about

the molecular mechanisms of these processes is exploding during the past few years and researchers are coming to a better understanding of the phenomenon. The challenge now is to utilize this knowledge with rational integration of plant's responses during plant-microbe interactions. Advent of newer molecular tools has given researchers a new ability to undertake plethora of studies in this endeavour. Cloning and characterization of disease resistance genes and identification of key regulatory genes in plant defense have ushered in a new horizon of prospects. Molecular markers hold promise in delineating the novel resistance genes and quantitative trait loci (QTL) at specific chromosomal locations. Novel genes across the species barriers are being introduced to create resistant transgenics through genetic engineering.

Molecular diagnostics

Biodiagnosis using indicator plants and serodiagnosis through serological agglutination are old yet effective tests for viruses occurring in high concentration. During the last few decades several novel diagnostic techniques with increased sensitivity have been developed (Please see review by Khurana & Garg 1993). Some of these are briefly described in Table 1.

Table 1. Some of the modern sensitive pathogen detection techniques

Enzyme Linked Immunosorbent Assay (ELISA)	Enzyme-linked immunosorbent assay (ELISA) is a very sensitive test that determines the presence of an antigen (e.g. a plant virus) by means of an enzymatic reaction. It is based on the fact that α -globulin can be linked to an enzyme without losing its immunological properties and enzyme activity. Usually α -globulin is first bound to the solid phase (polystyrene plate or even nitrocellulose membrane) on which the sample is 'trapped', i.e. by an antibody linked with an enzyme. It is suitable for testing a large number of samples at a time without requiring large quantities of expensive antisera and chemicals. Commercial ELISA 'kits' for different viruses are available now.
Indirect-ELISA	When enzyme is linked to the antibody (primary antibody) for ELISA, the test is termed as 'Direct ELISA', but if it is linked to another molecule, which detects the primary antibody, the test is called 'Indirect ELISA'. It is advantageous employing two

species of antibodies, i.e. coating antibody is from one animal species (e.g. sheep, goat and chicken) while the second antibody is from another animal species (e.g., rabbit) or vice-versa to reduce the background signals and amplify the positive reactions. It also helps in establishing serological relationship among diverse strains of the same virus or between different viruses.

DAS-ELISA

Double antibody sandwich-ELISA protocol, developed in 1977 has been widely adopted with minor variations. This technique is about 4-10 times sensitive than other detection/tests for potato viruses X and S.

DOT-ELISA (dot immunobinding ELISA)

This is similar to standard das-ELISA except that it employs nylon or nitro-cellulose membranes in place of microtitre plate. Other types of membranes used in dot-ELISA include polyvinylidene difluoride (PVDF) membrane or diazobenzylmethyl (DBM) or diazophenylthioether (DPT) modified cellulose papers with the ability to trap negatively charged proteins. The technique is sensitive, quick and handy for field-testing and can be used even if spotted membranes need to be mailed to some laboratory for probing. The developed membranes can also be dried for showing the results later or stored as 'records'. A rapid field dot-ELISA version is commercially available now as Spot Check™ from ADGEN, UK.

Radio-immunosorbent assay (RIA)

A modified form of ELISA employing ¹²⁵I labelled IgG as the second antibody, which is done in microtitre plates like ELISA and das-ELISA except that the bound labelled antibody is eluted by acidification for quantification of bound ¹²⁵I. The radioactivity measured is proportional to the amount of virus in the sample. It is more sensitive than the standard das-ELISA and has least variability in results. But due to hazards of radioisotopes, RIA is not commonly used in plant virology.

**Enzyme Linked
Fluorescent Assay
(ELFA)**

This is a modification of ELISA where the substrate is replaced with a chemical, which yields a fluorescent product that can be read with a fluorimeter. The assay enhances detection of several antigens and involves use of highly specific antibodies against the target antigen. Normally for plant viruses using alkaline phosphatase based ELISA the substrate employed is 4-methyumbelliferyl phosphate (MUP). The solid phase used is either frosted polystyrene beads of 3 to 6 mm dia or Immulon I or Immulon II plates. Another variant of ELFA known as time-resolved fluoroimmuno assay is 50-100 times sensitive than standard ELISA. It is possible to detect even PLRV in single aphid. Micro-das ELISA and magnetic microsphere based ELISA are now available to economise on antibody/conjugate, incubation time and labour yet maintaining high sensitivity of the test. Protein-A sandwich-ELISA (PAS-ELISA) and In-direct avidin-biotin complex-ELISA (indirect ABC-ELISA) are also important versions developed to have enhanced sensitivity of assays. Commercially available or custom-made kits of these two types have made them popular world over especially in laboratories related to virus resistance breeding.

**Recombinant
Antibody ELISA
(R-Ab-ELISA)**

This is the latest serodiagnostic technique utilising the bacterially expressed recombinant antibodies as fragments of variable heavy and light chains of antibodies (scFv). After selection of the specific scFv, recombinant scFv fused with different proteins, including alkaline phosphatase (AP) with a greater enzyme activity can be produced for use in ELISA. It is useful in cases of viruses where polyclonal antibody production is not easy. The major advantage of R-Ab is the indefinite storability of the scFv encoding DNA and production of uniform quality antisera within 2-4 weeks through

culture of *Escherichia coli* in an economical manner as against expensive specialized animal cell cultures needing ultra-freezing facilities essential for monoclonal antibody production.

**Immunosorbent
Electron Microscopy
(ISEM)**

This technique was first described in 1973. It is a highly sensitive method for virus detection and is either at par or better than ELISA. Plant virus diagnosis through ISEM can be carried out by any one or more of the following methods (i) immunosorption (ii) decoration and (iii) clumping. ISEM was observed to be 100 times more sensitive than the conventional leaf dip electron microscopy for detection of PLRV. A simpler technique involving antibody clumped virions trapped on to protein A treated grids (called Protein A Linked Immunoelectron Microscopy or PALEM) for detecting PLRV was also standardized at the Central Potato Research Institute, Shimla.

**ISEM through
Immunogold
Labelling**

Colloidal gold particles are electron opaque and thus act as very good markers when conjugated with antibodies. Antibody labelled gold particles are particularly useful when (i) a specific virus is to be identified in a mixture of viruses or (ii) when defined virus-particles fail to form (as in the case of defective tomato spotted wilt virus isolates) and only nucleoprotein aggregates occur in the host cell. Even more than one type of virus in a mixture can be identified by using a mixture of gold particles of different size coated with different viral antibodies. Antibody coated gold particles are also used to detect viral antigens *in situ* within the virus-infected host cells. Colloidal gold particles of wide range of sizes (from about 5nm to over 50 nm) can be easily prepared.

**Monoclonal
Antibodies**

are highly epitope specific and have been used extensively to differentiate strains of a viruses especially for epiphytotic studies and/or to determine the profile(s) of virus(es) infecting a particular crop variety in any area. They also check

or reduce the background in ELISA and help maintain an unending supply of specific antibodies which may be used either singly or in combination(s) as required.

Nucleodiagnosis
or Molecular
Indexing

Detection of nucleic acids of the infectious agents is possible through hybridization tests. It is done by spotting them on to a nitrocellulose membrane followed by hybridization with the labelled probes, 'tailored' specifically against targeted pathogen. *Nucleic Acid Spot Hybridization (NASH)*: Viroids lack a protein coat and hence can not be detected by immunological techniques. The nucleic acid hybridization is based on reassociation of two homologous complementary strands of nucleic acids to form a double stranded DNA or RNA and their detection. In all such cases, a single stranded nucleic acid probe which has been labelled either with a radioisotope (^{32}P or ^{35}S) or affinity tags (biotin), is 'hybridized' with homologous sequences which can be visualized and quantified. Development of the NASH test for viroid was the result of continued investigations on the molecular nature/mechanism of viroid replication and pathogenesis. Since its initial report in 1981, practical diagnostic NASH procedure for PSTVd has undergone several modifications, especially the substitution of single stranded complementary RNA probes with double stranded cDNA probes. Use of the RNA probes help avoid non-specific binding of the probe to the nitrocellulose membrane, through a simple post hybridization incubation with pancreatic RNase. The 'cold' or non-radioactive (biotin-labelled) nucleic acid probes have longer shelf life, low cost and safety in use but the samples need to be purified by phenol extraction and their sensitivity in practice is not much greater than that of ELISA. One can detect as low as 1pg DNA per sample spot. The nucleic acid probes were also applied for detection of other viruses having coat protein. Nucleic acid probes hold great promise for detection of low concentration of virus infections in plants/tissues

below the limits of serological detection. A number of PVX strains/serotypes have been found to have differences in specificity of hybridization because of differences in their coat protein structure. These can also help in understanding the molecular mechanism involved in the breaking of PVX resistance in hybrids due to PVX-^{HB} strain or for the virus(es) that are either difficult to purify or poorly antigenic. Multiplex detection is also possible using this technique.

Polymerase Chain
Reaction

The polymerase chain reaction (PCR) has the ability to amplify a specific DNA fragment or cDNA transcript in an impure mixture by means of a simple automated reaction. It is possible to amplify specific DNA sequences measuring 50 bp to over 10,000 bp in length in an automated DNA thermal cycler. PCR has been able to get rid of the common limitations or disadvantages of NASH methods *yet allowing easier testing, starting from very small sample quantities, enhanced sensitivity and easy interpretation of the results.* RNA can also be used as a template for amplification following the synthesis of complementary cDNA using reverse transcriptase. The amplified DNA is detected by procedures such as staining with ethidium bromide or silver nitrate after agarose or polyacrylamide gel electrophoresis or by hybridisation with labeled probes. This can be effectively used for multi-virus or multi-strain detection in a single test. PCR has proved useful in the identification and diagnosis of several viroids, viruses, and mycoplasma-like organisms directly from nucleic acid extracts of infected hosts. The technique can also be used for detection of bacterial/fungal pathogens.

Immuno-Capture
Reverse
Transcriptase
Polymerase Chain
Reaction
(IC-RT-PCR):

This is the latest, highly sensitive and most reliable detection test making use of specific antibodies to first capture the virions and then detection through RT-PCR. It is particularly suitable for detection in case of extremely low virus titre in the host.

In fact, RT-PCR, IC-RT-PCR and R-Ab-ELISA are the future alternatives to the common serological tests. They may be adopted routinely provided simple yet affordable protocols are developed in near future.

Mechanism of plant disease resistance

The range of phytopathogenic organisms that attack plants is diverse and each has a unique mode of pathogenicity. Despite the vast array of pathogens, resistance is the rule and susceptibility is the exception. The majority of potential pathogens are stopped before they infect, while, majority of them simply can not start their lifecycles on most plants. When the pathogen is in principle capable of initiating infection, plants have at least two sets of genetically defined overlapping defense modes that they can deploy. These responses reduce the range of plants that pathogen can infect. The first is an inducible defense system (host-resistance) that results from race specific pathogen recognition by the product of a dominant or semi-dominant plant resistance (*R*) gene with a product derived from the corresponding dominant pathogen avirulence (*Avr*) gene (Dangl & Jones 2001). The second is a basal defense system that limits the growth of virulent pathogens – called general defense system or non-host resistance (Heath 2000). A thorough understanding of molecular mechanisms of plant disease resistance is pre-requisite for any manipulation of this economically important trait. An overview of resistance mechanisms with special emphasis on resistance genes have been presented below.

Induced defense response or host resistance

Induced defense responses are characteristic features of incompatible gene-for-gene interactions associated with disease resistance and are primarily manifested in the form of hypersensitive response (HR). Hypersensitive reaction is expressed by localized induced cell death in the host plant at the site of infection. The genetic basis of HR-mediated disease resistance was first clarified by Flor (1971), who demonstrated that the resistance of flax to the fungal pathogen, *Melampsora lini*, was a consequence of gene-for-gene interactions of pathogen *Avr* genes and their corresponding plant *R* genes. In gene-for-gene interactions, R proteins (act as receptor protein) determine recognition of specific elicitors, encoded directly or indirectly by avirulence genes, resulting activation of a cascade for host genes that leads to HR and inhibition of pathogen growth (Ji *et al.* 1998). Physiological features of HR common to a plant's response to different pathogens include a rapid oxidative burst; ion fluxes characterized by K^+ - H^+ -exchange; cellular decompartmentalization; cross linking and strengthening of plant cell wall; production of antimicrobial compounds;

and induction of pathogenesis related (PR) proteins such as chitinases, glucanases and osmotins (Gilchrist 1998). HR subsequently triggers a defense response, referred to as systemic acquired resistance (SAR) that acts non-specifically throughout the plant through induction of diffusible signal (Ryals *et al.* 1996). Salicylic acid and its derivatives are the most potential candidates to participate in the systemic signaling pathway that induces SAR (Feys & Parker 2000).

Molecular cloning and characterization of R genes

Because of their importance in engineering crops for disease resistance considerable efforts have been made for cloning of R genes from diverse plant species since 1992. Over the last few years numerous plant R genes were cloned and characterized.

The first plant R gene to be cloned was the maize *Hm1* gene. This gene, which controls resistance to race-1 isolate of *Cochliobolus carbonum*, was identified by transposon tagging with *Mu* transposon. *Hm1* encodes a NADPH-dependent HC-toxin reductase (HRTC). HRTC inactivates HC-toxin, a pathogenicity factor produced by the fungus *C. carbonum* Nelson race-1 that permits the fungus to infect certain genotypes of maize (Johal & Briggs 1992). The genetics of the interaction between maize and *C. carbonum* differ from that of gene-for-gene systems because toxin-deficient strains of this fungus lose ability to cause disease in maize cultivars that do not carry *Hm1*. Similarly, three other R genes that conform to classic gene-for-gene relation were cloned by transposon tagging using heterologous corn elements, *Ac/Ds*. These genes were: tomato *Cf-9* gene conferring resistance to the fungal pathogen *Cladosporium fulvum* (Jones *et al.* 1994); flax *L6* gene conferring resistance to the fungal pathogen *Melampsora lini* (Lawrence *et al.* 1995); and tobacco *N* gene for resistance to tobacco mosaic virus (Whitham *et al.* 1994). Most of the R-genes have been isolated by map-based cloning. Isolation proceeded through the well-defined steps of precise genetic localization, saturation of the region with markers, isolation of large yeast artificial chromosome (YAC) clones, and identification of cDNAs from the area and finally complementation.

R proteins can be grouped into five classes based on the structural features. The first class has cytoplasmic receptor like proteins that contain leucine-rich repeats (LRR) involved in elicitor binding and a nucleotide binding site (NBS). The family of R proteins with NBS-LRR motif includes RPS2, RPP5 and RPM1 from *Arabidopsis*; Prf and I2 from tomato; N from tobacco; and L⁶ and M from flax. Besides LRR and NBS domains, N, L⁶ and RPP5 have NH₂-terminal domains with homology to cytoplasmic domains of the *Drosophila* developmental protein Toll and the mammalian immune response interleukin-1 receptor (1L-1R). RPS2, RPM1 and Prf

proteins contain a putative leucine Zipper (LZ) motif at their NH₂-terminus (Staskawicz *et al.* 1995). The second class of R protein includes Pto that falls under serine-threonine protein kinase family. The third class includes Cf-2 and Cf-9 that has putative transmembrane receptors with large extra-cytoplasmic LRR domains (TM-LRR). The fourth class is represented by the rice R protein Xa21. Xa21 has a putative transmembrane receptor with an extracellular LRR domain and an intracellular serine-threonine kinase domain. The Xa21 structure suggests an evolutionary link between LRR protein (Cf) and the Pto kinase. The fifth class, represented by Hm1 is distinct from other R proteins because no corresponding Avr protein is involved in its function (Young 2000). List of cloned R genes and structural features of their proteins are given in Table 2.

Table 2. A summary of cloning and characterization of plant R genes.

Class	R Gene	Plant	Pathogen	Avr gene	Structure
1	<i>RSP2</i>	<i>Arabidopsis</i>	<i>Pseudomonas syringae</i> pv. <i>tomato</i>	<i>AvrRpt2</i>	LZ-NBS-LRR
	<i>RPM1</i>	<i>Arabidopsis</i>	<i>P. syringae</i> pv. <i>maculicola</i>	<i>AvrRpm1</i> , <i>avrB</i>	LZ-NBS-LRR
	<i>Prf</i>	Tomato	<i>P. syringae</i> pv. <i>tomato</i>	<i>AvrPto</i>	LZ-NBS-LRR
	<i>Mla1</i>	Barley	<i>Blumeria graminis</i> f sp <i>hordei</i>	<i>AvrMla1</i>	LZ-NBS-LRR
	<i>N</i>	Tobacco	Tobacco mosaic virus	Unknown	TIR-NBS-LRR
	<i>L⁶</i>	Flax	<i>Malampsora lini</i>	<i>AL⁶</i>	TIR-NBS-LRR
	<i>M</i>	Flax	<i>M. lini</i>	<i>AM</i>	TIR-NBS-LRR
	<i>RPP5</i>	<i>Arabidopsis</i>	<i>Peronospora parasitica</i>	<i>AvrPp5</i>	TIR-NBS-LRR
	<i>I2</i>	Tomato	<i>Fusarium oxysporum</i>	Unknown	NBS-LRR
	<i>Pi-ta</i>	Rice	<i>Magnaporthe grisea</i>	<i>AvrPita</i>	NBS-LRR (?)
	<i>RPW 8</i>	<i>Arabidopsis</i>	<i>Erysiphe cruciferarum</i> , <i>E. cichoracearum</i>	Unknown	NBS-LRR (?)
	<i>Ctv</i>	<i>Poncirus trifoliata</i>	Citrus tristeza virus	---	NBS-LRR
2	<i>Pto</i>	Tomato	<i>P. syringae</i> pv. <i>tomato</i>	<i>AvrPto</i>	Protein kinase
3	<i>Cf-9</i>	Tomato	<i>Cladosporium fulvum</i>	<i>Avr9</i>	LRR-TM
	<i>Cf-2</i>	Tomato	<i>C. fulvum</i>	<i>Avr2</i>	LRR-TM
	<i>Cf-4</i>	Tomato	<i>C. fulvum</i>	<i>Avr4</i>	LRR-TM
4	<i>Xa21</i>	Rice	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	Unknown	LRR, Protein kinase
5	<i>Hm1</i>	Maize	<i>Cochliobolus carbonum</i> (race 1)	None	Toxin reductase

Non-host resistance

Non-host resistance is the most common form of disease resistance exhibited by plants (Heath 2000). This is a resistance shown by an entire plant species to a specific parasite or pathogen. Generally, nonspecific elicitors secreted by pathogen are the prime inducers of defense responses in non-host plant-pathogen interactions. There is considerable evidence that pre-formed defenses (constitutive) are a major component of non-host resistance, particularly in non-domesticated plants. Plants contain pre-formed peptides, proteins, and non-proteinaceous secondary metabolites that are potentially deterrents against microbial infection (Morrisey & Osbourn 1999). Hypersensitive cell death similar to that induced by host resistance mechanism is also the frequent expression of non-host resistance. Plant cells commonly respond to elicitors or microbial pathogens with an oxidative burst during which reactive oxygen species (ROS) are generated extra-cellularly, causing hypersensitive cell death, which is now universally accepted as a form of programmed cell death (PCD) (Lam *et al.* 2000). It is assumed that a number of plant cell death promoting gene families (PCD genes) trigger cell death during PCD. For fungi that try to penetrate directly into epidermal cells, considerable evidence points to the cell wall as a primary site of non-host resistance expression. Wall-associated defenses include the peroxidative cross-linking of phenolic compounds fueled by ROS generation. Deposition of other substances such as silica and callose-containing papillae can also combine to produce physical barriers to infection (Gilchrist 1998).

Protein kinases, elements of the mitogen-activated protein (MAP) kinase pathway and protein phosphatases are thought to be involved in signal transduction cascades triggered by specific and nonspecific elicitors (Desikan *et al.* 1999). Details of how elicitor from *Phytophthora sojae* interacts with parsley leaves and cells for induction of non-host defense responses has been demonstrated using *in situ* hybridization and transient expression studies. A nucleus-located zinc-finger-type transcription activator (WRKY 1) was shown to mediate fungal elicitor-induced gene expression. WRKY1 gets activated by phosphorylation, and binds to the palindromically positioned W-boxes (that function as a rapid-acting elicitor-response element) to express *PR-1* target gene (Eulgem *et al.* 1999). This is the first *in vivo* functional characterization of the regulation of part of a nonspecific elicitor-induced gene activation cascade.

Genomics and disease resistant genes

An interesting observation on number and organization of disease resistance genes was made from genomic sequencing of *Arabidopsis* and

rice. The complete *Arabidopsis* sequence permits a comprehensive analysis of the diversity of NB-LRR *R*-gene sequences in one plant. Annotation revealed ~150 sequences with homology to the NB-LRR class of *R* genes. *R* homologues are unevenly distributed between chromosomes, with 49 on chromosome 1, 2 on chromosome 2, 16 on chromosome 3, 28 on chromosome 4, and 55 on chromosome 5. Despite the fact that many previously isolated *R* genes seem to reside in local multigene families, there are 46 singleton *Arabidopsis* *R* gene homologues, 25 doublets, 7 loci with three copies, and individual loci with four, five, seven, eight and nine NB-LRR-encoding genes. Thus, *Arabidopsis* has ~100 *R* loci distributed over all the chromosomes (Meyer *et al.* 1999; Dangl & Jones 2001). Analysis of rice BAC end-sequences representing ~5% of the rice genome reveals probable presence of 750-1500 NBS-encoding genes in rice. All NBS-encoding genes in rice encode non-TIR type NBS, TIR-type genes have not been detected in genomic or expressed sequence tag (EST) sequences from any grass species (Meyer *et al.* 1999).

So far, only a few clusters of resistance genes have been sequenced. The complete sequencing of the *RPP5* cluster in *Arabidopsis*; *Cf-4/9* and *Pto* cluster in tomato; and partial sequencing of the *Dm3* cluster in lettuce revealed highly duplicated regions containing little more than resistance-gene homologues. The *RPP5* cluster contains 8-10 homologues spread over ~90 Kb, interspersed with protein kinase pseudogenes and retrotransposones (Noel *et al.* 1999). The *Cf-4/9* cluster contains five resistance genes spread over 36 Kb and interspersed with fragments of *lox* genes, which may have played a role in the duplication of the region (Parniske *et al.* 1997). Five *Pto* homologues are found to be spread over 60 Kb along with a single NBS-LRR gene (*Prf*) that is necessary for the function of at least two members of the *Pto* cluster (Salmeron *et al.* 1996). The *Dm3* region in lettuce is the largest resistance gene locus characterized at the molecular level so far. In this gene at least 24 resistance gene homologues are spread over at least 3.5 Mb (Meyer *et al.* 1998).

Genes that have altered expression in compatible and incompatible plant-pathogen interactions have also been targeted for characterization by microarray analysis (Baldwin *et al.* 1999). These analyses have provided comprehensive data on expression profiles, both for genes already implicated in plant-pathogen interactions as well as for many genes that were not previously known to be involved in resistance or susceptibility. Moreover, the analysis of global expression profiles of a large number of plant genes in response to infection and treatment with signal molecules

will provide a base to identify commonalities among defense pathways. Recently, changes in the expression patterns of 2,375 ESTs were examined simultaneously by cDNA microarray analysis in *Arabidopsis* after inoculation with an incompatible fungal pathogen *Alternaria brassicicola* or treatment with defense-related signaling molecules SA, MJ or ethylene. A comparison of expression profiles revealed induction of 126 genes by multiple treatments that are likely to be regulated by the same or overlapping defense signaling pathways indicating the existence of a substantial network of regulatory interactions and coordination during plant defense among the different defense signaling pathways (Schenk *et al.* 2000). However, the function of individual gene has to be confirmed on a gene-by-gene basis through high-throughput reverse genetics approach, virus-induced gene-silencing or viral over-expression as well as the use of gene knockout libraries and promoter-trap strategies.

Deployment of plant-pathogen interaction for disease management

The growing understanding of the diverse molecular mechanisms underlying the reactions of plants to pathogen infection holds promise for future progress in the development of strategies to engineer plants with enhanced resistance (Stuiver & Custers 2001). Visible manifestation of the progress in this domain are cloning and characterization of many genes for disease resistance and their downstream effectors including SAR, bacterial pathogenecity factors, elicitors, toxins etc.

Plant genomics offers new tools so that novel disease resistance gene of broad-spectrum resistance can be looked for. Analysis of EST libraries demonstrated that plants such as *Arabidopsis* and soybean express hundreds of potential *R*-genes (Botella *et al.* 1997). Many such genes have already been mapped to genetically characterized resistance loci in a variety of plant systems, including *Arabidopsis*, potato, soybean, lettuce, maize, wheat etc. Obviously, the optimized discovery of *R*-genes in both domesticated and exotic germplasm will be of paramount importance in the future and these genes can be rapidly transferred into advanced commercial genotypes. One such gene is pepper *Bs2* conferring resistance against *Xanthomonas campestris*. The Avr protein recognized by *Bs2* is not only produced by pathovars of *X. campestris* that infect pepper but also by pathovars that infect hosts such as tomato, brassica and citrus. *Bs2* has recently been cloned and expression of this gene in tomato confers resistance against bacterial spot disease caused by *X. campestris* pv. *vesicatoria* (Tai *et al.* 1999).

Attempts have been made to broaden and optimize the activities of *R*-proteins through genetic manipulations of *R*-genes. Wild-type tomato

Pto gene confers resistance only to certain races of *Pseudomonas syringae* pv. *tomato* that contain the Avr gene *AvrPto*. Replacement of the weak endogenous promoter of *Pto* with strong CaMV promoter resulted in not only increased resistance to Pst (*avrPto*) but also a partial control of unrelated pathogens such as *X. campestris* and *C. fulvum* (Tang *et al.* 1999). Studies on structure and function of R-protein demonstrated that LRR region is involved in R-gene specificity. Ellis *et al.* (1999) demonstrated that exchange of LRR region can result in recognition of a different spectrum of pathogens than that of the original R-gene. Further studies on various domains of R-genes may make it possible to optimize the efficacy and durability of R-genes.

Because of the durability of non-host resistance over time, it is commonly speculated that non-host resistance could be exploited to improve disease resistance within host species. The non-host resistance of tobacco against the potato pathogen *Phytophthora infestans* is correlated with the ability of tobacco to respond hypersensitivity to an elicitor of this pathogen. The product of a tobacco gene that shares similarities with R genes was indeed recently reported to interact with a 10 kDa peptide elicitor (INF1) produced by *P. infestans* (Keen *et al.* 1999). Similarly, in parsley a factor required for active non-host responses is 100 kDa plasma membrane protein that binds to a cell wall glycoprotein elicitor of the soybean pathogen *P. sojae*. This elicitor receptor has recently been isolated biochemically (Nennstiel *et al.* 1998). Transfer of such non-host genes to susceptible hosts may have a tremendous impact on the ability to control aggressive pathogens.

Knowledge on molecular mechanism of elicitor-receptor binding and consequent induction of hypersensitive reaction in host plants provides the researchers a new tool for elicitor-dependent artificial induction of broad-spectrum defense response. In one of the approaches, broad-spectrum disease resistance has been generated by the introduction of an elicitor gene coupled to a pathogen-inducible promoter. Transgenic tobacco plants were generated harbouring a fusion between the pathogen-inducible tobacco *hsr 203J* gene promoter and a *P. cryptogea* gene encoding highly active elicitor cryptogein. Under non-induced conditions the transgene was silent but upon infection by virulent pathogen, cryptogein production was stimulated which coincided with the fast induction of several defense genes at and around the infection sites. Induced elicitor production resulted in a localized necrosis that restricted further growth of the pathogen. The transgenic plants displayed enhanced resistance to fungal pathogens that were unrelated to *Phytophthora* species, such as *Thielaviopsis brassicola*, *Erysiphe cichoracearum* and *Botrytis cinerea* (Keller *et al.* 1999).

Plant pathogens often produce non-specific toxins that are involved in the development of disease symptoms. Engineering plants for resistance to toxins produced by pathogens represents an elegant way to prevent the damage caused by infection. A good source for toxin resistant genes is the toxin-producing organisms themselves. Tabtoxin produced by *P. syringae* pv. *tabaci* during infection provokes chlorosis by inhibition of glutamine synthetase, resulting in the accumulation of toxic levels of ammonia. In *P. syringae* pv. *tabaci*, the gene *ttr* (tabtoxin resistance) encodes an acetylating enzyme that inactivates the tabtoxin. The cloned gene has been expressed in tobacco and the resulting transgenic plants developed complete resistance to the bacteria (Anzai *et al.* 1989). Similarly, phaseolotoxin, produced by *P. syringae* pv. *phaseolicola*, is responsible for chlorosis on plants through the irreversible inhibition of the chloroplast enzyme ornithine carbamoyltransferase (Oetase). Resistance of the bacterium to its own toxin is in part attributed to the presence of an Oetase isozyme, encoded by the gene *argK* that is insensitive to the toxin (Mosqueda *et al.* 1990). Transformation of tobacco with *argK*, rendered the plants insensitive to chlorosis induced by purified toxin. Recently, transgenic sugarcane has been developed against leaf scald disease following this approach. Xylem invading pathogen *Xanthomonas albilineans* causes leaf scald disease of sugarcane. It produces a family of low molecular weight toxins (albicidins) that selectively block prokaryote DNA replication and cause characteristic chlorotic symptoms by blocking chloroplast development. Albicidin detoxifying gene (*alb*) cloned from *Pantoea dispersa* (biocontrol against leaf scald disease) provided resistance against leaf scald disease in sugarcane when expressed under the control of maize ubiquitin promoter (Zhang *et al.* 1999).

The genes involved in SAR response have also been targeted as potential candidates for manipulation towards resistance management. One such key SAR signaling gene is *Myb1* induced by tobacco mosaic virus in resistant tobacco plants. *Myb1* encodes a transcription factor that binds to a promoter element of the pathogenesis-related gene *PR1a*. Modification of *Myb1* expression levels in transgenic tobacco plants was shown to increase resistance against both TMV and *Rhizoctonia solani* pathogen (Yang & Klessig 1996). Mutant screening in *Arabidopsis* revealed two genes, *cpr6* and *Ssi1* that trigger not only genes associated with SAR but also genes involved in jasmonic acid activated disease response pathway (Shah *et al.* 1999). Although these two genes are yet to be cloned, it is assumed that *cpr6* and *Ssi1* are involved as switches modulating cross-talk between different defense pathways. A gene, *Pad4*, encoding a protein

with homology to lipases has been cloned from *Arabidopsis* and shown to enhance signals that activate plant defenses (Jirage *et al.* 1999). Inactivation of *pad4* leads to extreme susceptibility against a wide variety of pathogens including *Erysiphe orontii*, *Peronospora parasitica* and *P. syringae*. Thus overexpression of *Pad4* in transgenic plants may enhance disease resistance signaling and may prove exceptionally interesting for the development of broad-spectrum disease resistance. Genetic analysis of SAR in *Arabidopsis* led to the cloning of the *NIM1/NPRI* (noninducible immunity/nonexpressor of PR) gene mutations in which SAR induction is abolished. The NIM1/NPRI protein shows similarity to NF- κ B and I- κ B factors controlling numerous cellular responses in mammalian systems (Ryals *et al.* 1997).

Other pathways leading to expression of defense genes (thionin *etc.*) other than PR genes had also been the focus of research. For example, infection of *Arabidopsis* with necrotrophs such as *Alternaria brassicola* leads to induction of thionin and defensin-like genes such as *PDF1.2* but does not really result in PR-1 induction (Van Wees *et al.* 1997). Overexpression of the thionin gene in *Arabidopsis* leads to partial resistance against *Fusarium oxysporium* (Epple *et al.* 1997) indicating that non-SAR pathways may also be useful for disease resistance engineering.

Gene mapping and marker-assisted selection

The power to select desirable individuals based on genotypic configuration is an extremely powerful application of DNA markers and gene mapping. DNA markers in genomic region of interest enable breeders to select plants on the basis of genotype rather than phenotype. This is highly helpful if scoring of disease resistance is cumbersome or the pathogen is absent. The advent of several DNA marker technologies allows entire genome to be assayed for linkage to disease resistance(s). This has recently resulted in the identification of an increasing number of DNA markers linked to resistance genes using a variety of approaches. Near-isogenic lines (NILs) and bulked segregant analysis (BSA) is being used increasingly for rapid mapping of monogenic resistance genes using segregating populations (Michelmore 1995). It is now relatively facile to saturate particular region of the genome with molecular markers using RFLP, RAPD, AFLP or microsatellites (Paterson 1996). Marker analyses have located many disease resistance genes in the genome of crop plants tightly linked to one or the other markers (Table 3). These linked markers will greatly assist in marker-aided selection and also facilitate gene pyramiding.

Table 3. Examples of mapping and identification of markers linked to disease resistance genes in important crop plants.

Host	Pathogen	Gene	Type of markers
Tomato	<i>Verticillium dahliae</i>	<i>Ve</i>	RAPD
	Yellow leaf curl virus	<i>Ty1</i>	RFLP
	<i>Oidium lycopersicum</i>	<i>O1</i>	RFLP, SCAR
Potato	<i>Phytophthora infestans</i>	<i>R1, 3, 6, 7</i>	RFLP
	Potato virus Y	<i>Ry</i>	SCAR, RFLP
	Potato virus X	<i>Rx1, Rx2</i>	RFLP
	Potato virus X	<i>Nb</i>	AFLP, CAPS
	Potato virus X	<i>Nx_{phu}</i>	RFLP
Lettuce	<i>Bremia lactucae</i>	<i>Dm17, 18</i>	RAPD/SCAR
	<i>Plasmopora lactucae-radiciis</i>	<i>plr</i>	RAPD, RFLP
	Turnip mosaic virus	<i>Tu</i>	RAPD, RFLP
Soybean	Soybean	<i>Rsv</i>	SSR, RFLP
Common bean	<i>Uromyces appendiculatus</i>	<i>Up2</i>	RAPD
	Common bean mosaic virus	<i>I</i>	RAPD
Pea	Pea seed-borne mosaic virus	<i>sbm-1</i>	RFLP, RAPD
	Pea common mosaic virus	<i>Mo</i>	RFLP
Barley	Barley yellow mosaic virus	<i>Ym4</i>	RFLP
	<i>Erysiphe graminis</i> f.sp. <i>hordei</i>	<i>Mla</i>	RFLP
Wheat	<i>Puccinia recondita</i>	<i>Lr9</i>	RAPD
	<i>Puccinia striiformis</i>	<i>YrH52, Yr15</i>	AFLP, RAPD, Microsatellite
<i>Arabidopsis</i>	Cucumber mosaic virus	<i>RCY1</i>	CAPS

Genetically complex form of plant disease resistance governed by polygenes is studied through QTL (quantitative trait loci) mapping based on DNA markers. QTL mapping helps in (i) describing roles of resistance loci, (ii) assessing race specificity of partial resistance genes, and (iii) interaction between resistance genes. Plant development and the environment can also be analyzed using QTLs. Over the last few years QTL mapping has dissected quantitative resistance to a number of pathogens. The examples include resistance to rice blast fungus, late blight of potato, gray leaf spot of maize and bacterial wilt of tomato (Table 4). Plant disease resistance gene (R-gene) homologues are normally present in multiple copies in the plant genome and they can be used as candidates at QTLs for resistance to plant diseases. Such studies will eventually provide an entry point for the most ambitious goal of positional cloning of partial resistance genes known only by small and continuous effects on phenotype (Yano 2001).

Table 4. QTLs for disease resistance in some crop plants.

Host	Disease (Pathogen)	Number of QRLs*	Observations
Barley	Powdery mildew (<i>Erysiphe graminis</i>)	2	No evidence of QRL at the genomic location of <i>Mla12</i>
Common bean	Bacterial blight (<i>Xanthomonas campestris</i>)	7	One QRL mapped to same interval as <i>Rhizobium</i> nodule number locus
Maize	Grey leaf spot (<i>Cercospora zeae-maydis</i>)	> 10	One QRL significant in all populations and environments
Mungbean	Powdery mildew (<i>Erysiphe polygoni</i>)	3	Two QRLs significant 65 days post-inoculation; third QRL at 86 days
Pea	Blight (<i>Ascochyta pisi</i>)	3	Resistant allele from susceptible parent associated with transgressive segregation
Potato	Late blight (<i>Phytophthora infestans</i>)	11	One QRL was race specific; Two QRLs in same region as major resistance loci
	Late blight (<i>P. infestans</i>)	---	Co-localisation of QTL for late blight resistance, vigour and earliness
Rice	Blast (<i>Pyricularia oryzae</i>)	10	Three QRLs mapped to same regions as qualitative resistance loci
Tomato	Bacterial wilt (<i>Pseudomonas solanaceum</i>)	3	Effect of QRLs on phenotype varied according to inoculation method
Tomato	Yellow leaf curl virus	3	A major QRL located vary near <i>Mi</i> , root knot nematode resistance

*Quantitative Resistant Loci

DNA markers make it possible to select individuals or lines with crossing-over very near to the gene of interest, thereby, removing linkage-drag that frequently comes from a donor parent. Markers can also be used to select individuals with a minimum undesirable donor genes in the regions unlinked to an introgressed segment in a backcrossing. This possibility may be especially helpful in accessing useful gene pool from wild relatives of agriculturally important crops (de Vicente & Tanksley 1993).

In the special case of disease-resistance, marker-assisted breeding has special roles. The introgression of disease resistance genes (both monogenic and polygenic) into elite cultivars via traditional breeding can take up to 15-20 years. Marker-assisted breeding programmes have been estimated to reduce the breeding time by about 50-70%. Moreover, pyramiding several major resistance genes into valuable genetic background

is simplified through the use of marker-based selection (Tanksley *et al.* 1989). This would be especially helpful when screening for one resistance gene interferes with the ability to screen for another. An example of a multidisciplinary effort to identify and characterize important traits including disease resistances using molecular markers is the NABGMP (North American Barley Genome Mapping Project). Similar efforts are also ongoing in all major crops.

Disease resistant transgenics

Although classical breeding led to the development of high yielding and biotic stress resistant varieties of almost all crops and significantly improved food production, it generally remains a "hit or miss" technique. Genetic engineering or genetic transformation has two distinct advantages over classical breeding. The first is selectivity. In classical breeding the entire genome of parent plants is recombined, thereby requiring several cycles of backcrossing and selection to eliminate undesirable traits. On the contrary, genetic transformation can introduce a single gene for desired trait without disturbing plant's genetic make-up. Secondly, the classical breeding is confined to only cross-fertile plants, and thus can exploit limited variability. Recent developments in molecular biology and genetic transformation have made it possible to identify, isolate and transfer any desirable gene from any living organism to plants and vice versa. This has brought the whole genetic diversity of living world together, which was unthinkable some 25 years ago.

Three major discoveries in molecular biology made it possible to move a gene from one organism to another. The first was the discovery and description of genetic material DNA. Now it is known that gene is a piece of DNA, which carries out a specific function by producing specific protein and also that the structure and function of DNA is similar in all living organisms. The second discovery was that some bacterial DNA is in the form of free floating rings called "plasmids". In nature bacteria often exchange plasmids. Therefore, plasmids were thought to be ideal carriers of new genes. A naturally occurring soil-borne bacterium *Agrobacterium tumefaciens* can transfer a piece of its Ti plasmid into plant cells causing crown gall disease. This plasmid (after removing crown gall disease causing genes) is used to carry new (desired) genes into dicotyledonous plants. But how to cut a single gene and fix it into plasmids? This was made possible by third discovery of special enzymes "restriction endonucleases" and "ligase". There are more than 500 restriction endonucleases, which cut DNA molecule at specific sites. If same restriction enzyme is used to cut foreign gene and to cut open the plasmid, the cut ends of the new gene and

plasmid are complementary and chemically "sticky". DNA ligase seals such sticky ends to form a duplex DNA chain.

Strategies used for developing disease resistant transgenics in some of the crops are briefly described below.

Viral resistance

Viral diseases cause alarming losses to crop productivity either by reduction in yield or quality of the harvest. In case of vegetatively propagated crops viral disease is of serious concern as all the clones obtained from infected plants will carry the infection deteriorating the quality of seed material. Management of this disease is much difficult because of complex disease cycles, efficient system of transmission and non-availability of chemical control. The successful biotechnological strategies include expression of genes either derived from pathogen (pathogen-derived resistance, PDR) that would disrupt normal balance between viral products and/or viral movement and completion of life cycle, or from foreign source (antiviral protein or compounds) that would prevent virus multiplication and spread by interacting with either incoming viral genome or their expression products or by killing the host cell (Kundu & Mandal 2001). The best documented approach for generating virus resistant transgenic plants is coat protein (CP)-mediated resistance. The CP gene of tobacco mosaic virus (TMV) was used in the first demonstration of virus-derived resistance in transgenic plants (Powell-Abel *et al.* 1986). Since then CP-gene approach has been successfully employed against many viruses in several crops. Second approach is transgenic expression of genes encoding the viral RNA-dependent RNA polymerase that is generally referred to as replicase-mediated resistance (Thomas *et al.* 2000). Third approach is engineering of negative mutant form of movement protein (MP) to interfere with viral movement, which is an important step in viral infection (Cooper *et al.* 1995). The added advantage of expression of dysfunctional MP is that it confers resistance to several other viruses also (Table 5).

Attempts have also been made to develop viral resistant transgenic plants through expression of transgene (encoding antiviral protein or compound) which is not part of the viral genome. The ability of ribozyme to cleave target RNA has been utilized to develop citrus exocortis viroid

(CEVd)-resistant transgenic plants (Atkins *et al.* 1995). Recently, a hammerhead ribozyme targeting the mRNA of rice dwarf virus (RDV) was constructed and when introduced into rice variety displayed high resistance against viral symptoms (Han *et al.* 2000). Expression of double stranded RNA-specific ribonuclease has been used to impart resistance against viroids, which encode none of the proteins necessary to support their own replication but rely on host machinery. The single stranded circular RNA of the viroid when forms a double stranded RNA like structure (ds RNA) during replication gets destroyed by the ribonuclease. Following this strategy potato spindle tuber viroid (PSTV) resistant transgenic potato has been developed by expressing yeast dsRNA-specific ribonuclease (Sano *et al.* 1997). Pokeweed (*Phytolacca americana*) antiviral protein (PAP) has ribosome-inhibiting properties. PAP kills host cell in the vicinity of viral infection and restricts spread of virus providing broad spectrum resistance. A similar kind of antiviral protein (PIP), which shares 84% homology with PAP has been isolated from cDNA library of *P. insularis*. Recombinant PIP synthesized in *Escherichia coli* inhibits protein synthesis *in vitro*. Constitutive expression of PIP in transgenic potato results in broad-spectrum resistance against PVX, PVY and PLRV (Moon *et al.* 1997). The plantibody approach, which is one of the most recent innovations aims ectopic expression of genes encoding antibodies and offers a potential tool to block viral infectivity (Schillberg *et al.* 2001). This has been successfully demonstrated in tobacco. Examples of virus transgenics are given in Table 5.

Fungal resistance

Many of the fungi have chitin and glucan as major structural polysaccharides in their cell wall. Plants coevolved natural defense mechanisms through enzymatic degradation of these components by endochitinase and gucanases. Expression of these enzymes is induced in host plants in response to pathogen attack to degrade fungal cell wall (Strittmatter *et al.* 1998). Therefore, different classes of chitinases and gucanases of diverse source have been engineered into a number of transgenic plants with varied degree of success against fungal pathogens. In *Brassica napus*, the constitutive synthesis of a chimeric endochitinase encoded by a fusion between a tomato and a tobacco chitinase gene provided field tolerance to *Cylindrosporium concentricum*, *Sclerotinia sclerotiorum*, and *Phoma lingam*, each causing economically important diseases (Grison

Table 5. Strategies for the development of viral resistance and some examples of transgenic plants tolerant to viruses.

Strategy	Inhibited stages	Mode of infection	Virus*	Transgenic plant
<i>Pathogen-derived resistance</i>				
Coat protein	Uncoating	Competition for RNA	TMV PEMV PLRV PhMV	Tobacco Pea Potato Tomato
Replicase	Replication	Competition for enzyme	PVY PSbMV RYMV PLRV	Potato Pea Rice Potato
Movement protein	Transport	Interference with transport	TMV PLRV, PVX, PVY	Tobacco Potato
Helper component	Transport	Interference with transport	TEV	Tobacco
Viral protease	Replication	Polyprotein processing	PVY	Potato
Antisense RNA	Translation	Blocks viral RNA and prevents Translation	PLRV	Potato
Satellite RNA	Assembly	Compete for capsids	CMV	Tobacco
<i>Antiviral proteins</i>				
Ribozyme	Translation	Cleaves viral RNA	CEVd RDV	Tomato Rice
Ribonuclease	-----	Degrades ds-RNA (viroids)	PSTV	Potato
2',5'-oligoadenylate synthetase	Replication	Degrades ds-RNA	CMV	Tobacco
Pokeweed antiviral protein	Translation	Inhibits rRNA of 60S subunit	TMV, PVX	Tobacco
Phytolacca insularis antiviral protein	Translation	Inhibits polypeptide synthesis through N-glycosidase activity	PVX, PVY, PLRV	Potato
Plantibodies	Assembly	Competes for viral protein against which it is generated	AMCV TMV	<i>Nicotiana benthamiana</i> Tobacco

*AMCV = artichoke mottle crinkle virus; CEVd = citrus exocortis viroid; CMV = cucumber mosaic virus; PhMV = Physalis mottle virus; PEMV = pea enation mosaic virus; PLRV = potato leaf roll virus; PSbMV = pea seed-borne mosaic virus; PSTV = potato spindle tuber viroid; PVX = potato virus X; PVY = potato virus Y; RDV = rice dwarf virus; RYMV = rice yellow mottle virus; TEV = tobacco etch virus; TMV = tobacco mosaic virus; and TNV = tobacco necrosis virus.

et al. 1996). Class II catalase has been shown to bind salicylic acid and activate SAR in tobacco. Expression of tobacco class II catalase gene in transgenic potato plants leads to constitutive expression of endogenous catalase homologue gene and is associated with enhanced resistance to *P. infestans* (Yu *et al.* 1999). Similarly, other pathogenesis-related (PR) genes, such as a PR1a gene or an osmotin gene from tobacco, have been proven to mediate enhanced resistance to *Phytophthora* species when constitutively expressed in tobacco or potato (Liu *et al.* 1994). In another approach, truncated osmotin gene has been designed through removal of C-terminal leader sequence to ensure its expression in the extracellular matrix and better protection against invading fungal hyphae. Transgenic potato plants expressing this truncated osmotin gene exhibited high level of resistance to *P. infestans* (Liu *et al.* 1996).

Ribosome-inactivating proteins (RIP) from plants inhibit translation in distantly related species including fungus by N-glycosidic cleavage of 28S rRNA. As a consequence, elongation factor 2 (EF 2) binds less efficiently and the elongation step in protein synthesis is inhibited (Cornelissen & Melchers 1993). Expression of barley type 1 RIP gene under the control of potato wound inducible promoter in transgenic tobacco demonstrated resistance to *Rhizoctonia solani* (Logemann *et al.* 1992). Other antifungal proteins that have been successfully employed against fungal pathogens are pokeweed antiviral protein and defensin. Pokeweed antiviral protein has been demonstrated to have antifungal activity also. Expression of this protein in transgenic tobacco confers resistance to *R. solani* (Wang *et al.* 1998). Plant defensins are a small group of cysteine-rich antifungal proteins that express systemically during fungal pathogen infection. Constitutive expression of *Raphanus sativus* defensin gene, Rs-AFP2, in tobacco confers resistance to the foliar pathogen *Alternaria longipes* (Terras *et al.* 1995). Similarly, AFP cDNA from alfalfa (alfAFP) provided protection from *Verticillium dahliae* in potato (Gao *et al.* 2000).

Phytoalexin, a class of compounds (typical example is stilbenes from *Vitis vinifera* and *Picea sitchensis*) accumulate in host plants in response to pathogen attack. These have been shown to involve in defense of many plant species against fungal attack. Transformation and expression of stilbene synthase gene from *V. vinifera* in potato has been shown to substantially reduce damage after *P. infestans* infection. During the pathogen attack host plant often displays localized cell death. Induction of artificial cell death by genetic engineering in response to pathogen attack is one of the viable strategies that have been employed successfully against a number of fungal diseases. A bacterial ribonuclease gene (barnase) from *Bacillus*

amyloliquefaciens and its inhibitor barstar were introduced into potato under the control of fungal-infection-specific (prp1-1) and CaMV 35S promoters, respectively (Strittmatter *et al.* 1995). The dual mode of regulation ensures higher expression of barnase in the close vicinity of infection sites, thereby leading to cell death. Transient accumulation of active oxygen species (referred to as oxidative burst), such as H₂O₂, superoxide radical (O₂⁻) and the hydroxyl radical (OH⁻), is one of the most rapid responses in plants after pathogen attack (Baker & Orlandi 1995). The enzyme glucose oxidase from *Aspergillus niger* catalyzes the oxidation of b-D-glucose by molecular oxygen, yielding gluconic acid and H₂O₂. Constitutive expression of the gene encoding glucose oxidase in transgenic potato resulted in resistance development against *P. infestans* (Wu *et al.* 1995). Recently a novel strategy of simulating the presence of a pathogen through lesion mimic phenotype and consequent induction of anti-pathogen defense mechanisms has been employed to enhance resistance of plants to fungal pathogens. Potato plants have been engineered to develop lesion-mimic phenotype through constitutive expression of a bacterio-opsin (bO) proton pump isolated from *Halobacterium halobium*. These plants had increased levels of salicylic acid and pathogenesis-related mRNAs, and displayed enhanced resistance to *P. infestans* (Abad *et al.* 1997).

Development of fungus resistant transgenics using different strategies is summarized in Table 6.

Bacterial resistance

There are only few reports on production of transgenic plants resistant to bacteria. The major approach employed against bacterial pathogens is production of antibacterial proteins that kill or retard the pathogen. Antibacterial proteins, which have already been introduced to develop bacterial resistance, include lytic peptides, lysozymes and iron sequestering glycoproteins (Strittmatter *et al.* 1998). Lytic peptides are small proteins (e.g. cecropin, attacin and their synthetic analogues) with an amphipathic α -helical structure whose effect is to form pores in bacterial membranes. Cecropins have been expressed in tobacco against *Ralstonia solanacearum* and *Pseudomonas. syringae* pv. *tabaci* and attacins in apple plants against *Erwinia amylovora*. Symptom reduction upto 50% after inoculation with *E. amylovora* was reported in apple. However, satisfactory levels of resistance could not be achieved in tobacco expressing cecropin B, probably because of degradation of cecropin by plant proteases. A synthetic analogue of cecropin, Shiva 1, had been introduced into potato

Table 6. A summary of fungal resistant transgenic plants in various crops.

Plant	Gene/Protein	Target pathogen
<i>PR proteins</i>		
Tobacco	Chitinase from <i>Serratia marcescens</i>	<i>Alternaria longipes</i>
	Chitinase from bean	<i>Rhizoctonia solani</i>
	Chitinase from <i>Nicotiana tabacum</i>	<i>R. solani</i>
	<i>PR-1a</i> gene from tobacco	<i>Phytophthora parasitica</i>
	Glucanase from alfalfa and chitinase from rice	<i>Cercospora nicotianae</i>
	Chitinase and glucanase from barley	<i>R. solani</i>
Rice	Chitinase from rice	<i>R. solani</i>
<i>Brassica napus</i>	Bean chitinase	<i>R. solani</i>
	Chimeric endochitinase by fusion of tomato and tobacco chitinase gene	<i>Cylindrosporium concentricum</i> , <i>Sclerotinia sclerotiorum</i> , <i>Phoma lingam</i>
Potato	β -1,3-endoglucanase from soybean	<i>P. infestans</i>
	Class II catalase from tobacco	<i>P. infestans</i>
Kiwifruit	β -1,3-endoglucanase from soybean	<i>Botrytis cinerea</i>
<i>Antifungal proteins</i>		
Tobacco	Barley RIP	<i>R. solani</i>
	Pokeweed antiviral protein II	<i>R. solani</i>
	Rs AFP-2 from Radish	<i>A. longipes</i>
Potato	AlfAFP from alfalfa	<i>Verticillium dahliae</i>
	Osmotin from tobacco	<i>P. infestans</i>
<i>Antifungal compounds</i>		
Potato	Stilbene synthase from grapevine	<i>P. infestans</i>
<i>Genes/proteins that induce artificial cell death</i>		
Potato	Barnase and barstar	<i>P. infestans</i>
	Glucose oxidase from <i>Aspergillus niger</i>	<i>P. infestans</i>
	Bacterio-opsin from <i>Halobacterium halobium</i>	<i>P. infestans</i>
	Bacterio-opsin from <i>H. halobium</i>	<i>P. infestans</i>
Tomato and Tobacco	Bacterio-opsin from <i>H. halobium</i>	<i>P. infestans</i>

to circumvent this problem. Transgenic plants showed delayed symptoms and reduced disease severity when inoculated with *Ralstonia solanacearum* (Watanabe *et al.* 1993). Similarly, transgenic potato plants of cv. Desiree expressing another synthetic cecropin gene, SB 37, when challenged with a virulent strain of *R. solanacearum* showed resistance comparable to that of field-resistant cultivar Cruza 148 (Montanelli *et al.* 1995). Under combinational approach a chimeric gene fusing cecropin (from giant silk moth) and melittin (from honeybee venom) has been constructed. The gene was introduced into potato resulting in resistance to *Erwinia carotovora* induced soft rot (Osusky *et al.* 2000). Tachyplesin is another class of lytic

peptide isolated from the horse-shoe crab. Expression of a gene encoding tachyplesin I in transgenic potato resulted in reduced tuber rot caused by *Erwinia carotovora* (Allefs *et al.* 1996). Among the antibacterial peptides of non-plant origin that has been expressed in plants is lactoferrin. Lactoferrin is an iron-binding glycoprotein known to have antibacterial properties with unknown mechanism. The expression of a human lactoferrin gene in tobacco delayed disease development caused by *R. solanacearum* (Mourgues *et al.* 1998).

Lysozymes are ubiquitous enzymes with specific hydrolytic activities directed against the bacterial cell-wall peptidoglycan. The bacteriophage T4 lysozyme gene was engineered by fusing its coding sequence to the signal peptide sequence of the barley α -amylase gene to target the protein at the intercellular spaces. *Erwinia carotovora* enters and spreads via intercellular spaces. Introduction of this construct into potato resulted in low-level expression, but efficient secretion of the protein to the intracellular spaces accompanied by a marked increase in resistance to soft rot pathogen *E. carotovora* (During *et al.* 1993). Some of these examples are given in Table 7.

Table 7. Some examples of bacterial resistance transgenic plants.

Plant	Gene/Protein	Origin	Target
Apple	Attacin	Giant silk moth	<i>Erwinia amylovora</i>
Potato	Shiva 1 (synthetic cecropin B)	-----	<i>R. solanacearum</i>
	SB 37 (synthetic cecropin B)	-----	<i>R. solanacearum</i>
	Cecropin and melittin	Giant silk moth and honey bee venom, respectively	<i>E. carotovora</i>
	Tachyplesin	Horseshoe crab	<i>E. carotovora</i>
	Lysozyme	T4 bacteriophage	<i>E. carotovora</i>
	Glucose oxidase	<i>Aspergillus niger</i>	<i>E. carotovora</i>
Tobacco	Cecropin B	Giant silk moth	<i>Ralstonia solanacearum</i> <i>Pseudomonas syringae</i> pv. <i>tabaci</i>
	Lactoferrin	Human	<i>Rhizoctonia solani</i>
	Lysozyme	Human	<i>P. syringae</i> pv. <i>tabaci</i>
	Bacterio-opsin	<i>Holobacterium halibium</i>	<i>P. syringae</i> pv. <i>tabaci</i>

Selected references

- Abad M S, Hakimi S M, Kaniewski W K, Rommens C M, Shulaev V, Lam E & Shah D M 1997 *Mol. Plant-Microbe Interact.* 10 : 653-45.
- Allefs S J H M, Jong E R, Florack D E A, Hoogendoorn C & Stiekema W J 1996 *Mol. Breed.* 2 : 97-105.
- Anzai H, Yonetyama K & Yamaguchi I 1989 *Mol. Gen. Genet.* 219 : 492-94.
- Atkins D, Young M, Uzzell S, Kelly L, Fillatti J & Gerlach W L 1995 *J. Gen. Virol.* 16 : 1781-90.
- Baker C J & Orlandi E W 1995 *Annu. Rev. Phytopath.* 33 : 299-21.
- Baldwin D, Crane V & Douglas R 1999 *Curr. Opin. Plant Biol.* 2 : 96-103.
- Botella M A, Coleman M J, Hughes D E, Nishimura M T, Jones J D & Somerville S C 1997 *Plant J.* 12 : 1197-11.
- Cooper B, Lapidot M, Heick J A, Dodds J A & Beachy R N 1995 *Virology* 206 : 307-13.
- Cornelissen B J C & Melchers L S 1993 *Plant Physiol.* 101 : 709-12.
- Dangl J L & Jones J D G 2001 *Nature* 411 : 826-33.
- de Vicente M C & Tanksley S D 1993 *Genetics* 134 : 585-96.
- Desikan R, Clarke A, Atherfold P, Hancock J T & Neill S J 1999 *Planta* 210 : 97-103.
- During K, Porsch P, Fladung M & Lorz H 1993 *Plant J.* 3 : 587-98.
- Ellis J G, Lawrence G J, Luck J E & Dodds P N 1999 *Plant Cell* 11 : 495-506.
- Epple P, Apel K & Bohlmann H 1997 *Plant Cell* 9 : 509-20.
- Eulgem T, Rushton P J, Scmelzer E, Hahlbrock K & Somssich I E 1999 *EMBO J.* 18 : 4689-99.
- Feys B J & Parker J E 2000 *Trends Genet.* 16 : 449-55.
- Flor H H 1971 *Annu. Rev. Phytopathol.* 9 : 275-96.
- Gao A G, Hakimi S M, Mittanck C A, Wu Y, Woerner B M, Stark D M, Shah D M, Liang J & Rommens C M 2000 *Nat. Biotechnol.* 18 : 1307-10.
- Gilchrist D G 1998 *Annu. Rev. Phytopathol.* 36 : 393-414.
- Grisson R, Grezes-Besset B, Schneider M, Lucante N, Olsen L, Leguay J J & Toppan A 1996 *Nat. Biotechnol.* 14 : 643-46.
- Heath M C 2000 *Curr. Opin. Plant Biol.* 3 : 315-19.
- Ji C, Smith-Becker J & Keen N T 1998 *Curr. Opin. Biotechnol.* 9 : 202-207.
- Jirage D, Tootle T L, Reuber T L, Frost L N, Feys B J, Parker J E, Ausubel F M & Glazebrook J 1999 *Proc. Natl. Acad. Sci. USA.* 96 : 13583-88.

- Johal G S & Briggs S P 1992 *Science* 258 : 985-87.
- Jones D A, Thomas C M, Hammond-Kosack K E, Balint-Kurti P J & Jones J D G 1994 *Science* 266 : 789-93.
- Keen N, Gelvin S & Long S 1999 *Mol. Plant-Microbe Interact.* 12 : 835-38.
- Keller H, Pamboukdjian N, Ponchet M, Poupet A, Delon R, Verrier J L, Roby D & Ricci P 1999 *Plant Cell* 11 : 223-35.
- Khurana S M, Paul & Garg I D 1993 In: *Advances in Horticulture*. Vol.7. Potato. MPH, New Delhi, pp. 529-566.
- Kundu P & Mandal R K 2001 *Proc. Indian Natl. Sci. Acad.* B67 : 53-80.
- Lam E, Kato N & Lawton M 2001 *Nature* 411 : 848-53.
- Lawrence G J, Finnegan E J, Ayliffe M A & Ellis J G 1995 *Plant Cell* 7 : 1195-1206.
- Liu D, Raghothama K G, Hasegawa P M & Bressan R A 1994 *Proc. Natl. Acad. Sci. USA* 91 : 1888-92.
- Logemann J, Jach G, Tommerup H, Mundy J & Schell J 1992 *Biotechnol.* 10 : 305-308.
- Meyer B C, Chin D B, Shen K A, Sivaramakrishnan S, Lavelle D O, Zhang Z & Michelmore R W 1998 *Plant Cell* 10 : 1817-32.
- Meyer B C, Dickerman A W, Michelmore R W, Sivaramakrishnan S, Sobral B W & Yong N D 1999 *Plant J.* 20 : 317-32.
- Michelmore M W 1995 *Annu. Rev. Phytopathol.* 15 : 393-27.
- Montanelli C, Stetanini F M, Chiari Achiari T & Nascari G 1995 *Potato Res.* 38 : 371-78.
- Morrisey J P & Osbourn A E 1999 *Microbiol. Mol. Biol. Rev.*: 708-24.
- Mosqueda G, van den Brock G, Saucedo O, Bailey A M, Alvarez-Morales A & Herrera-Estrella L 1990 *Mol. Gen. Genet.* 222 : 461-66.
- Mourgues F, Brisset M N & Chevreau E 1998 *Trends Biotechnol.* 16 : 203-10.
- Nennstiel D, Scheel D & Nummerger T 1998 *FEBS Lett.* 431 : 405-10.
- Noel L, Moores T L, van der Biezen E A, Parniske M, Daniels M J, Parker J E & Jones J D 1999 *Plant Cell* 11 : 2099-12.
- Osusky M, Zhou G, Osuska L, Hancock R E, Kay W W & Misra S 2000 *Nat. Biotechnol.* 18 : 1162-66.
- Parniske M, Hammond-Kosack K E, Golstein C, Thomas C M, Jones D A, Harrison K, Wulff B B & Jones J D 1997 *Cell* 91 : 821-32.

- Paterson A H 1996 *Genome Mapping in Plants*, Texas: Academic Press.
- Powell-Abel P, Nelson R S, De B, Hoffman N, Rogers S G, Praley R T & Beachy R N 1986 *Science* 232 : 138-43.
- Ryals J, Weymann K, Lawton K, Friedrich L, Ellis D, Steiner H Y, Johnson J, Delancy T P, Jesse T, Vos P & Uknes S 1997 *Plant Cell* 9 : 425-39.
- Ryals J A, Neuenschwander U H, Willits M G, Molina A, Steiner H Y & Hunt M D 1996 *Plant Cell* 8 : 1809-19.
- Salmcron J M, Oldroyd G E D, Rommens C M T, Scofield S R, Kim H S, Lavelle D T, Dahlbeck D & Staskawic Z 1996 *Cell* 86 : 123-33.
- Schenk P M, Kazan K, Wilson I, Anderson J P, Richmond T, Somerville S C & Manners J M 2000 *Proc. Natl. Acad. Sci. USA* 97 : 11655-60.
- Shah J, Kachroo P & Klessig D F 1999 *Plant Cell* 11 : 191-206.
- Staskawicz B J, Ausubel F M, Bakaer B J, Ellis J G & Jones J D G 1995 *Science* 268 : 661-67.
- Strittmatter G, Goethals K & Van Montagu M 1998 In: *Subcellular Biochemistry*, New York: Plenum Press. pp. 191-213.
- Strittmatter G, Janssens J, Opsomer C & Botterman J 1995 *Bio/Technol.* 13 : 1085-89.
- Stuiver M H & Custers J H H V 2001 *Nature* 411 : 865-68.
- Tai T H, Dahlbeck D, Clark E T, Gajiwala P, Pasion R, Whalen M C, Stall R E & Staskawicz B J 1999 *Proc. Natl. Acad. Sci. USA* 96 : 14153-58.
- Tang X, Xie M, Kim Y J, Zhou J, Klessig D F & Martin G B 1999 *Plant Cell* 11 : 15-30.
- Tanksley S D, Young N D, Paterson A H & Bonierbale M W 1989 *Bio/Technol.* 7 : 257-64.
- Terras F R G, Eggermont K, Kovaleva V, Raikhel N V, Osborn R W, Kester A, Rees S B, Torrekens S, van Leuven F, Vanderleyden J, Cammue B P A & Broekaert W F 1995 *Plant Cell* 7 : 573-88.
- Thomas P E, Lawson E C, Zalewski J C, Reed G L & Kaniewski W K 2000 *Virus Res.* 71 : 49-62.
- Van Wees S C M, Pieterse C M J, Trijssenaar A, Van't Westende Y A M, Hartog F & Van Loon L C 1997 *Mol. Plant-Microbe Interact.* 10 : 716-24.
- Wang P, Zoubenko O & Tumer N E 1998 *Plant Mol. Biol.* 38 : 957-64.
- Watanabe K, Dodds J H, El-Nashaar H, Zambrano V, Benavides J, Buitron E, Siguenas C, Panta A, Salinsa R, Vega S & Golmirazaic A 1993 *Amer. Potato J.* 70 : 853-54.

- Whitham S, Dinesh-Kumar S P, Choi D, Hehl R, Corr C & Baker B 1994 *Cell* 78 : 1011-15.
- Wu G, Shortt B J, Lawrence E B, Levine E B, Fitzsimmons K C & Shah D M 1995 *Plant Cell* 7 : 1357-68.
- Yang Y & Klessig D F 1996 *Proc. Natl. Acad. Sci. USA* 93 : 14972-77.
- Yano M 2001 *Curr. Opin. Plant Biol.* 4 : 130-35.
- Young N D 2000 *Curr. Opin. Plant Biol.* 3 : 285-90.
- Yu D, Xie Z, Chen C, Fan B & Chen Z 1999 *Plant Mol. Biol.* 39 : 477-88.
- Zhang L, Ku J & Birch R G 1999 *Nat. Biotechnol.* 17 : 1021-24.

2

Bioinformatics packages for sequence analysis and practical applications

Dilip K Arora & Bandamaravuri Kishore Babu

In a field which has been dominated by structural biology for the last 2-3 decades, we are now witnessing a dramatic change of focus towards sequence analysis, spurred on by the advent of the genome projects and the resultant sequence deposit. The amount of sequence information available from public database is exponentially increasing. By 2005, 100 gigabases of sequences were deposited into GenBank. Advanced sequencing technologies and model organism genome projects, including human, were the major driving forces behind the sequence information explosion during the last decade. The prime challenge of bioinformatics is the rationalization of the mass of sequence information, with a view not only to derive more efficient means of data storage, but also to design more incisive tools.

The greatest challenge facing the molecular community today is to make sense of the wealth of data that has been produced by the genome-sequencing projects. Traditional molecular biology research was carried out entirely at the experimental laboratory bench but the huge increase in the scale of data being produced in this genomic era has aroused a need to incorporate computers into the research process.

Today's biological researches require parallel strategies to simultaneously gather, examine and integrate the large volumes of information. Biologists often face genome-wide or cross-genome analysis of genes of interest. Thus, without good data handling skills, researchers can not achieve their ultimate research goals. This is where biology requires informatics. Bioinformatics is a new field that examines complex biological data on the basis of statistics and computer science. It provides a powerful tool for collecting, maintaining, distributing and analyzing huge amounts of genome data. It also contributes to give biological meaning in the data by discovering structural and functional relationships to explain biological phenomena.

Biological and molecular

Biological databases are archives of consistent data that are stored in a uniform and efficient manner. These databases contain data from a broad spectrum of molecular biology areas. Primary or archived databases contain information and annotation of DNA and protein sequences, DNA and protein expression profiles. Secondary and derived databases are so called because they contain the result of analysis on the primary resources including information on sequence, patterns or motifs, variants and mutation and evolutionary relationships.

The purposes of biological databases are

- To make information available globally
- To make biological data available to scientists
- To systematize results from biological experiments and analysis
- To avoid duplication of experiments
- Consistency, accuracy and non-redundancy of data.

The most important general sequence databases are those hosted at the European Bioinformatics Institute (EBI) at Hinxton, UK; GenBank at Bethesda, MA, USA and DNA Data Bank of Japan (DDBJ). These three databases exchange the data submitted to them with each other on a daily basis. Each sequence, when submitted to a database, is assigned a unique identifier (accession number). All three databases, is assigned a unique identifier (accession number). All three databases will use the same accession number for that sequence. However, database structure, data accession data formats differ remarkably.

European Bioinformatics Institute (EBI) databases

A variety of general and specialized databases are mentioned at EBI. These include databases for primary structure collections, i.e. European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database, the protein databases SWISS-PORT and TrEMBL, amalgamated into the Universal Protein Resource UniPort (<http://www.ebi.uniprot.org/index.shtml>) in addition to higher order structure databases available in the Macromolecular Structure Database (MSD). Information on these and other data collections can be inspected at <http://www.ebi.ac.uk/Databases>. The detailed description is given in the *EMBL Nucleotide Sequence Database User Manual* which can be inspected at <http://www.ebi.ac.uk/enbl/Documentation/user/manual/userman.html>.

National Center for Biotechnology Information (NCBI)

NCBI was established in 1988 as a division of the National Library of Medicine (NLM) at the National Institute of Health (NIH). Its mission is defined as development, distribution and maintenance of various molecular databases and computer software in order to support biological and biomedical studies at the molecular level.

NCBI Sequence Submission System: GenBank provides two programs to support sequence submission.

- a. *BankIt* (<http://www.ncbi.nlm.nih.gov/BankIt/>) is a web based sequence submission tool that can be used for depositing a few sequences when annotation is not complicated. *BankIt* does not require any special tools to submit sequences other than web browser and the submission directions are fairly easy to follow.
- b. *Sequin* is a stand-alone program to submit and update long complex sequences and annotation information. Submission with *Sequin* provides sophisticated tools to review and verify the sequence and annotation before submission. Submission is finished by sending the *Sequin* output file (.sqn file) via e-mail to GenBank (<http://www.ncbi.nlm.nih.gov/Sequin/index.html>).

Sequence retrieval: NCBI's Entrez is an integrated database retrieval system. Its cross-reference system allows researchers to not only access nucleotide, protein or genome information but also related research articles and relevant records from 22 databases using text based query (<http://www.ncbi.nlm.nih.gov/Entrez/index.html>).

Sequence analysis tools at NCBI: The NCBI provides a wide range of biological data analysis tools that fall into the following major categories:

- a. *FASTA:* is an international format for storing biosequences. Program is available over the web or by download. FASTA searches molecular databases, the first line begins with ">" and a short description followed by the sequence. Current FASTA programs can be found at URL <http://fasta.bioch.virginia.edu/>.
- b. *BLAST:* Basic Local Alignment Search Tool is the most popular local alignment program for similarity search and sequence alignment developed by NCBI (Fig. 1). BLAST algorithm generates a list of short word match (default words size is 3 for protein and 11 for nucleotide) in query sequences and then database is searched for the occurrence of these words. Stand-alone BLAST search can be performed at any local

computer by installing the BLAST software. Stand-alone BLAST reduces searching time significantly by avoiding on-line communication and allows batch blast (submit multiple queries at once) against local databases downloaded from GenBank or created by user. Stand-alone BLAST document including installation can be found at NCBI's FTP site (<ftp://ftp.ncbi.nlm.nih.gov/blast/documents/blast.txt>).

NCBI BLAST

nucleotide-nucleotide Protein Translations Protein results for an BL

Query Sequence

TTGAGAAAAGTTGAGAAAGGTTGCTCGGGGGGGGACGGCCTTACGCTCCGAAAGCGAGGT
 TATTCTACTACGGCTTGAGGCLAGACGCCACCGCCGAGGCTTTGAGGGCGGCGCCAL
 GGACGGTGCCCAATACCAAGCAGAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATG
 CCCCCGGAATACCAAGGGGGCCAAATGCGCTTCAAGATTCGATTCATCGAATTC
 GCAATTCACATTACTTATCGCAATTCGCTCGCGTTCATCGATGCCAGAACCAGAGG

Search

Set subsequence From: To:

Choose database: nr

Now BLAST! or Run locally

Submit button

Options for advanced blasting

Limit by entry query or select from: fungi [EPR04]

Choose filter Low complexity Human repeats Mask for lookup table only Mask lower c

Fig.1. Part of BLASTn search form

Several BLAST programs are available based on search purpose and also based on query and database relation. Various forms of BLAST search programs are given below.

Program	Description
BLASTN	- Compare nucleotide query sequences against nucleotide databases.
BLASTP	- Compare amino acid sequence query against amino acid sequence database.
BLASTX	- Compares a nucleotide query sequence translated in all reading frames against a protein sequence database
TBLASTN	- Compares a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames

- TBLASTX - Compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database
- PHI-BLAST - (Pattern-Hit Initiated) Designed to search for proteins that contain a pattern in the query specified by the user.
- PSI-BLAST - (Position-Specific Iterated) Designed to find very distantly related protein sequences using a PSSM (position-specific scoring matrix) generated from each progressive search
- c. *BLAT*: stands for BLAST-like alignment tool that is designed to effectively align EST sequence to genomic sequences. Its algorithm is similar to BLAST in which it finds words match and extends to high scoring pairs (HSP). However, BLAT makes index of database first and then searches query. Moreover, it extends alignments on any number of perfect or nearly perfect hits and provides a large alignment. Web search is available at <http://genome.ucsc.edu/cgi-bin/hgBlat> and stand-alone is also available at <http://www.soe.ucsc.edu/~kent/exe/>.

Pairwise and multiple sequence alignments

Pairwise sequence alignment tries to find the optimal alignment in parts of sequences (local alignment) or in entire sequences (global alignment). In a global alignment, all of the nucleotides or amino acids in both sequences participate in the alignment, thus it is useful for aligning closely-related sequences. Local alignment finds and aligns related regions within sequences. It is more flexible than global alignment, which is useful to identify related regions that appear in a different order in two sequences.

Multiple sequence alignment is an extension of pairwise alignment to identify common regions within several sequences given as an input. This tool is mostly used for building phylogenetic trees and also creating sequence profiles which can be used to search distant related sequences in database.

CLUSTALW is a popular program for multiple sequence alignment. Both progressive global and local alignments can be done in *ClustalW*. The user has the option to control parameters to make the best alignments (e.g., word size, matrix, gap open, extension, etc.). It also provides two guide phylogenetic trees, cladogram (equal length of branched tree showing common ancestry) or phylogram (unequal length of branched tree showing evolutionary distances). Alignment can be further edited using Jalview program (<http://www.ebi.ac.uk/jalview/>).

Sequence assembler

Two well known methods are applied to generate genome sequences. One is called clone by clone (CBC) approach and other is whole genome shotgun (WGS) method. Both methods create many small overlapping sequences or reads, which are eventually assembled by computer software to build the sequence contigs.

Phred/phrap/consed package

- a. *Phred* software calls bases by reading electropherogram or trace files as raw data and assign the quality value to each base.
- b. *Phrap* is a program for assembling shotgun sequences based on sequence overlap. (<http://www.genome.washington.edu/UWGC/analysisistools/Phrap.cfm>)
- c. *Consed* is a program for viewing and editing phrap assembly in its finishing phase. It shows global assembly view with forward-reverse pairs, read depth and repeat match. Independent from phrap assembly, Consed also allows breaking or joining contigs by comparing contigs. *In silico* digestions can be generated and compared with real digestions to verify the overall assembly in final finishing. For primer walk in finishing phase, Consed provides built-in primer picking function.

Whole genome assembler

Whole genome assembly is a challenging procedure because it processes hundreds of thousands of reads at a time so repeat sequences possibly cause mis-assembly. Development for WGS assembler thus focuses on reducing computation time and resolving mis-assembly problems caused by repeat sequences in the genome.

- a. *PCAP* is a contig assembly program using parallel computer processors. First, it removes vector and low quality area from reads. Then, it uses BLAST2 to identify pairs of reads that contains potential overlaps. Identification of repetitive regions in reads is based on deep coverage by longer approximate matches. The score of every overlap is adjusted to reflect the depths of coverage for the two regions in the overlap. The consensus sequence of a contig is generated by constructing an alignment of reads in the contig. Human chromosome 20's assembly is simulated with PCAP and it showed N50 contig with scaffold length of 41 kb and 2Mb, respectively.
- b. *Phusion* program first read group sequences by determining the number of times that sequences of length k (called k -mer word) occur in the

data, and eliminates reads representing highly redundant *k*-mer sequences. It generates reads list and matrix based on reads showing less repetitive or unique *k*-mer distribution.

EST clustering

Expressed Sequence Tag (EST) is useful information in a sense that it is a profile of expressed gene sequences. It usually does not contain full length gene sequence because about 600 bp sequences are generated from 5' and 3' end of cDNA clones. At the same time EST permits low quality bases due to single pass sequencing and often some sequences are highly redundant in certain genes. In order to overcome these disadvantages and collect more unique sequences (called UniGene), clustering ESTs is necessary. *Phrap*, *TIGR* assembler and *CAP3* are used to cluster or assemble EST data.

Gene prediction

Gene prediction is one of the important subjects in genome projects. In eukaryotes, gene prediction and annotation is not a simple process mainly because of the various sizes of introns (uncoding sequences) located between exons (coding sequences). In addition, many genes have alternative splice variants. In other words, every eukaryotic gene shows different structures and length to be predicted.

Mainly three groups of gene prediction programs have been developed for genome wide annotation.

Ab initio program: *FGENESH*, *Genscan*, *Grail*, *MZEF* and *HMMgene*.

Similarity based program: *CRASA*, *AAT* and *AgenDa*.

Combined methods: *GenomeScan*, *Procrustes* and *FGENESH+*.

Repeat identification

Repetitive sequences occupy a large portion of most eukaryotic genomes and are divided into tandem (including simple sequence repeats or SSRs) and interspersed repeats. Transposable elements (TEs), one of the interspersed repeats, are the most abundant in repeat family.

- a. **RepeatMasker** at <http://repeatmasker.org> is a widely used program to find interspersed repeats (LINEs, SINEs, LTRs and DNA elements), simple sequence repeats (SSRs) and low complexity regions in the sequences using similarity search against well defined repeat database. User defined repeat database can be used to search against in stand-alone RepeatMasker.

- b. RECON** allows *de novo* detection and classification of repeat family in genomic sequences. RECON algorithm detects and groups repeats in the genome sequences by blast itself and clusters them to a repeat family using multiple sequence alignment. This approach helps repeat annotation by determining repeat boundaries in genomic sequences and also enables identification of new repeat elements. RECON is available from <http://www.genetics.wustl.edu/eddy/recon/>.

Other programs

- a. PipMaker** is a tool to align two sequences and generates a percent identity plot (PIP) and dot plot as output. PipMaker analysis can be performed at <http://pipmaker.bx.psu.edu/pipmaker/>. zPicture program <http://zpicture.dcode.org/> provides more dynamic alignment and visualization of comparing two sequences.
- b. rVISTA**-Regulatory Vista is a computational tool to identify evolutionary conserved transcription factor binding sites (TFBSs) by multiple alignment of orthologous sequences followed by prediction of TFBSs using TRANSFAC database collected from eukaryotic transcription factors. rVISTA 2.0 is available at <http://rvista.dcode.org/>.
- c. MUMmer** is a tool that allows rapid alignment of two large nucleotide or protein sequences or even genome to genome for comparison. Software is available at <ftp://ftp.tigr.org/pub/software/MUMmer>.
- d. EMBOSS** stands for The European Molecular Biology Open Software Suite and is developed for the molecular biology community. Currently, more than 100 programs are available in EMBOSS package grouped by analysis functions such as alignment, display, edit, enzyme kinetics, nucleotide, protein analysis and phylogeny. Many more applications will be added in the near future. It is difficult to describe all the programs here but EMBOSS is mainly used for sequence alignment, restriction map, CpG island analysis, primer design, sequence extraction, sequence retrieval from database, codon usage analysis, protein motif analysis and many more applications. It runs on UNIX environment with command line mode (<http://www.hgmp.mrc.ac.uk/Software/EMBOSS/download.html>).

Some important web addresses for further information

NCBI (Pubmed)	http://www.ncbi.nlm.nih.gov/pubmed
NCBI (Genbank)	http://www.ncbi.nih.gov/Genbank
DDBJ	http://www.ddbj.nig.ac.jp

Infobiogen	http://www.infobiogen.fr/services/dbcat
SWISSPROT	http://www.expasy.ch
EMBL	http://www.edi.ac.uk
PDB (PROTEIN DATA BANK)	http://www.rcsb.org/pdb
PIR (Protein Information Resource)	http://www.pir.georgetown.edu
Munich information center for protein sequences	http://mips.gsf.de
Welcome Trust Sanger institute	http://www.sanger.ac.uk
The Institute for Genomic Research	http://www.tigr.org
Ribosomal Database Project	http://www.rbp.cme.msu.edu
European Ribosomal RNA Database	http://www.rna.uia.ac.be
Probe base	http://www.microbial-ecology.de
The ARB project	http://www.air-home.de

II

METHODS AND PROTOCOLS

Isolation techniques for soil borne fungi, bacteria and nematodes

R Suseela Bhai & Santhosh J Eapen

Soil harbors various types of micro flora and fauna such as viruses, bacteria, fungi, algae, protozoa, nematodes, worms and arthropods. They may be saprophytic or parasitic capable of living within or outside the cells of higher or lower forms of life. A culture containing a single unadulterated species of cells is called a pure culture.

Several different techniques are available to isolate and study microorganisms in pure culture. Several media also have to be employed for isolating targeted organisms.

Isolation of fungi and bacteria from soil

To isolate fungi and bacteria, representative soil samples have to be collected from different places in the field and representative or composite samples may be used. For composite samples, the samples should be collected at random and mixed thoroughly. Depending upon the objective of the studies, soil samples should be collected from different depths such as 0-8cm (surface layer), 8-15cm and 15-30 cm, respectively. The different layers of soil vary greatly in their microbial population. These may be due to the influence of rhizosphere, which is the portion of soil adjacent to the root system of the plant and is influenced by the root system. The collected samples have to be stored separately in sterilized bottles and have to be assayed for microbial population at the earliest or within 10-15 days.

Techniques used for isolation of microorganisms include serial dilution agar plate, Warcup soil plate, syringe inoculation, immersion tube, screened immersion plates, plate profile, hyphal isolation, soil washing, partial pre sterilization, soil sieving, floatation baiting etc. A single method cannot be used to count all the different types of fungi present in a given sample. To have a complete spectrum of fungi present, a sample has to be processed by a variety of techniques.

Though various methods are available to isolate and enumerate microorganisms, the serial dilution agar plating method or viable plate count method and Direct soil plate or Warcup soil plate method are two most

widely used methods for the fungal isolation. Serial dilution agar plating is the commonly used method for the isolation and enumeration of bacteria or actinomycetes from soil.

Serial dilution agar plating method

The serial dilution agar plating method is based upon the principle that when material containing the microorganism is cultured each viable microorganism will develop into a colony. The number of colonies appearing on the plates represents the number of living organisms present in the sample. This will merely give the number of organisms present in the soil sample which are able to produce visible colonies on the specific nutrient medium used. Many other organisms will not be evident because of the nature of isolation medium and the incubation temperature or fail to develop into distinct colonies because of the competition on the agar plate. Hence the media and incubation conditions may be modified accordingly to achieve the isolation of target microorganisms present in the soil.

Procedure : A known amount of the soil is suspended or agitated in a known volume of sterile water (1g soil in 9 ml to make the total volume to 10ml) to make a microbial suspension. The dilutions can also be made in 0.85% sodium chloride in place of sterile distilled water. Serial dilutions (10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} etc) are made by pipetting measured volumes into additional dilution blanks (1ml or 10ml from each dilution to 99ml or 90ml sterile water blank). Finally 1 ml aliquot of various dilutions is added to sterile Petri dishes to which are added 15 ml of the sterile, cool molten media. Upon solidification, the plates are incubated in an inverted position for 3-7 days at 25°C. The number of colonies appearing on dilution plates may be counted, single colonies sub cultured and purified for further studies (10^{-3} - 10^{-4} for fungi, 10^{-5} - 10^{-7} for bacteria).

Usually Nutrient Agar is used for bacterial isolation and Glycerol Yeast Agar for actinomycetes, and Czapek-Dox agar, Rose Bengal Agar or Sabouraud Agar medium supplemented with chlortetracycline or streptopenicillin 10µg/ml for fungi.

Direct soil plate or Warcup soil plate method

In this method soil samples are air dried and add 0.005- to 0.15g soil in 5 sterile Petri dishes with the help of a sterilized cooled loop or transfer needle. To this add 15-20ml molten cooled media supplemented with streptopenicillin and Rose Bengal (Rose Bengal Agar). Dispense the particle through out the medium by gentle rotation of the Petri plate and allow it to solidify. Then incubate the plates at 25°C in an inverted position

for 15 days. Observe for the appearance of the colonies after 3 days of incubation and continue till 15 days. Purify various fungal isolates these obtained for identification and maintain for future use.

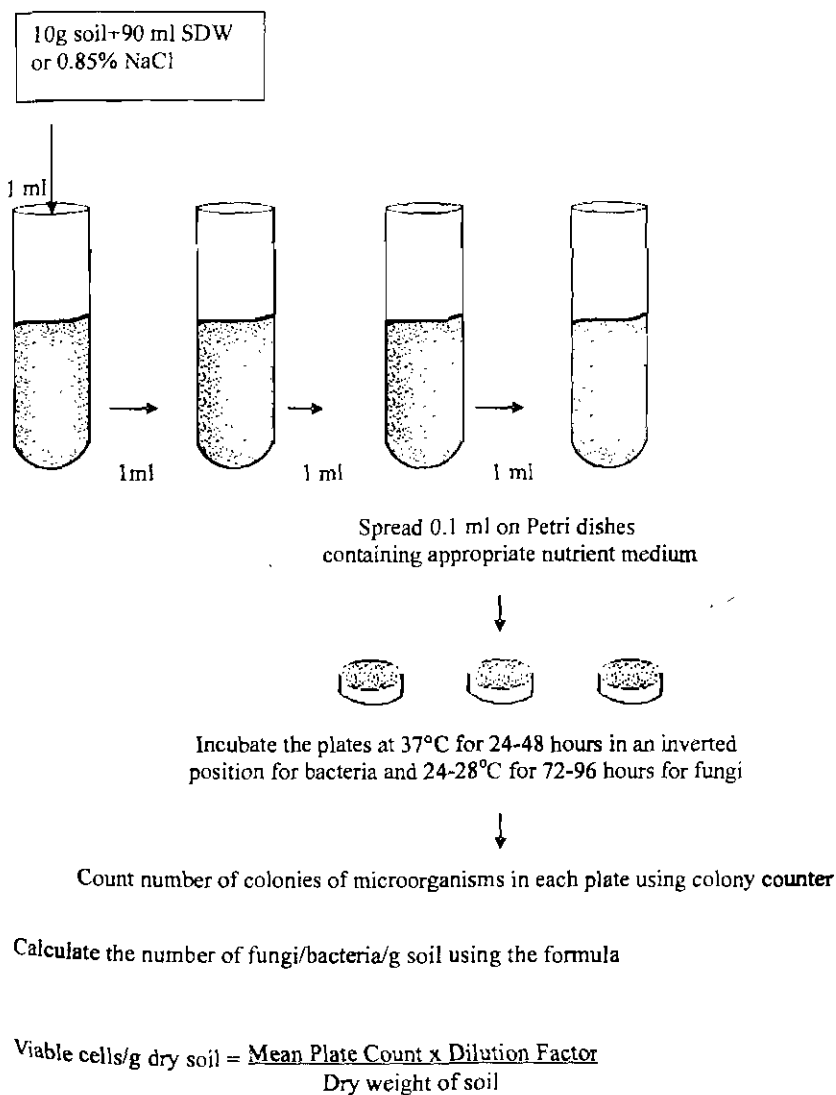


Fig 1. Serial dilution agar plate technique

SDW - Sterile Distilled Water

Soil also contains thermophilic bacteria particularly *Bacillus* sp. in varying numbers. Their presence and frequency can be estimated by incubating duplicate plates at 50-55°C for 24hr.

The number of heat resistant endospores in the soil sample may be estimated by holding 10^{-1} and 10^{-4} dilutions in a boiling water bath for two minutes. prior to plating out. The heat treatment will kill the majority of the organisms present with the exception of heat resistant spores.

Selective isolation

The isolation of specific group of microorganisms will necessitate using selective isolation media. So dilutions may be plated out in Czapek Dox agar to discourage growth of bacteria and encourage the development of fungi. The use of PDA adjusted to pH 4.5 will encourage the growth of yeasts and a tap water agar containing 1% (w/v) glycerol and 0.1%(w/v) asparagine will favor the development of actinomycete colonies. The number of azotobacter present in soil can be determined using Ashby's medium.

Selective media have been devised for the isolation of many pathogenic fungi, bacteria and actinomycete from the soil. Many rely on the use of various antibiotics or fungicidal substances to suppress bacteria or groups of fungi.

The carbon source experiment will also provide information on the wide range of carbon sources utilized by soil microorganisms. For eg. *Chromobacterium violaceum* from soil can be isolated using organisms ability to utilize undegraded starch. A few rice grains are scattered on the surface of moist soil in a Petri dish. The plate is incubated at room temperature for three to 5 days. Some of the rice grains turn to purple colour due to the growth of the deeply pigmented *C. violaceum*.

Isolation of fungi and bacteria from rhizosphere

The isolation procedures are same as for other soil sample except that the rhizosphere soil is taken for isolating organisms. To obtain rhizosphere soil, the soil is dig carefully to expose the lateral roots from targeted plant and the roots 2-5 cm from the tip are cut. They are tapped against the palm to remove as much of adhering soil as possible. Bits are transferred to 100 ml sterile water in 250 ml Erlenmeyer flasks. Sample in the flask is shaken for 5 minutes in rotary shaker or in hand for 3 minutes. Serial dilutions of the suspension are made with sterile water as explained elsewhere and plated in appropriate media to isolate the organisms. The portion of the suspension left over after plating is transferred to an

evaporating dish and dry weight of the solids is estimated. Microbial population per gram of dry rhizosphere sample is calculated.

Baiting

Plant material can be used as bait to isolate specific pathogens from the soil. Suitable baits include stem, leaves, surface sterilized roots, fruits etc. Seedlings also can be used as baits to detect the presence of specific pathogens. Baiting techniques are appropriate for zoosporic fungi as germinating sporangia produce zoospores that can actively move towards and infect the bait. The suitable baits can be applied to soil suspensions or to water in which pieces of diseased roots or leaves have been added in order to trap these fungi. For chitinous material it is customary to place the dry baits on top of the soil, which must be kept moist.

Eg: Techniques for the selective isolation of *Phytophthora* from the soil

A) Baiting: Take approximately 100cc (100g) of soil to be tested in a beaker. Add about 200ml of sterile water and mix it thoroughly using a glass rod. Put 10-20 leaflets of *Albizia*, incubate under humid conditions at 24-28°C for 72hr and observe for infection and confirm the presence of typical sporangia of *Phytophthora* under the microscope. The infected baits may be plated on PVPH media for isolation in pure culture.

Purification of microbial cultures

Purification generally involves the isolation of desired single spore colonies from a mixture. It can be done by taking a small portion of fungus on a culture loop and streaking it over an agar surface in a Petri dish. As the streak progresses the spore become more separated till individual colonies are obtained. This is also possible by making a suspension of spores in sterile water and streaking on agar. Another method of purification is using serial dilution plating. The inoculum is prepared in sterile water in test tubes and 1ml of the required final dilution is added to 15-20ml of melted agar cooled to about 40°C and poured into sterile Petri dishes and incubates.

Procedure: Growth of fungi obtained from soil dilution or by plating diseased specimens may be purified for further studies. Purification can be done by single spore isolation. A kit of the fungal growth is taken from the previously grown pure culture using a platinum loop (inoculation needle) and suspend in sterile water. Add 1ml of the suspension in a sterile petri plate and pour the agar media and rotate the plate to disperse the fungal particles. Incubate the plates at 24-28°C. The single spore colonies formed may be sub cultured to agar (PDA/CMA/PSA) slopes and maintain in the BOD or refrigerator for further studies.

Preservation and maintenance of cultures

Preservation techniques range from continuous growth through methods that reduce rates of metabolism to the ideal situation where metabolism is suspended. Many fungi that produce resistant or dormant structures that enable them to survive adverse conditions and these can be stored in the laboratory to retain viability of the organism. There are many methods available for the preservation and storage of fungi. Continuous growth techniques involve frequent transfer from depleted medium to fresh medium providing optimum growth conditions and there are also methods that delay the need to subculture. This method involves storage in a refrigerator, freezer (-10°C to -20°C), under a layer of oil or in water. Drying, usually of the resting stage such as spores or sclerotia, can be achieved by air drying in or above silica gel, in soil and by freeze drying.

1. Preservation on agar stands

Cultures are normally grown in test tubes or bottles on a nutrient medium, usually an agar gel with added nutrients. Many fungi can be maintained in this way for years. Most fungi can be grown on PCA or malt agar but some have specific growth requirements. Some fungi may need subculturing every 2-4 weeks, the majority in every 2-4 months, still others may survive for 12 months without transfer.

Cultures can be stored at room temperature in a cup board to protect the cultures from dust. But they may dry out rapidly in tropical climates and must be transferred to fresh media at least every six months. Storage at 4-7°C in a refrigerator or cold room can extend the transfer interval to 4-6 months from the average period of 2-4 months.

2. Preservation under mineral oil

Covering cultures on agar slants (0° to the horizontal) in 30ml universal bottles with mineral oil prevents dehydration and slows down the metabolic activity and growth through reduced oxygen tension. Mature healthy cultures are covered by 10mm of sterile mineral oil (liquid paraffin or medicinal paraffin Sp. gr. 0.830-0.890 sterilized by autoclaving twice at 121° C for 15 min.). If the oil is deeper than 10mm the fungus may not survive, whereas if the depth is less, exposed mycelium or agar on the sides of the container may allow moisture to evaporate and the culture to dry out.

Cultures can be retrieved from oil. This is done by removing a small amount of the colony on a mounted needle, draining away the oil as much

as possible and then streaking on to a suitable agar medium. Growth of this culture will be poor due to adhering oil, so sub-culturing is needed by re-isolating from the edge of the colony and transferring to fresh media.

3. Preservation in water

Agar blocks cut from the growing edge of the fungal colony are placed in sterile distilled water in Mc Cartney bottles and the lids are tightly screwed down. They can be stored at 20-25°C. Retrieval is by removal of a block and placing mycelium on a suitable growth medium. Any growth during storage in water can be reduced if the spores or hyphae are removed from the surface of agar media and no medium is transferred. *Phytophthora* and *Pythium* species can be stored in water for a period of 2-3 years without any loss of viability.

4. Preservation at ultra low temperature

Metabolism can be suspended by reducing the water availability in the cells by dehydration and freezing so that there is no medium for life processes to function. The temperature of the frozen material must be reduced below -70° to achieve condition where no physical or chemical reaction can occur requires storage below -139°C. An original culture should be preserved without sub culturing and a seed stock should be stored separately from the distribution stock. After preservation, the viability, spore germination, purity and identity should be rechecked and compared with data recorded before preservation.

Isolation of nematodes from soil

Nematodes are the most numerous multicellular animals on earth. A handful of soil will contain thousands of the microscopic nematodes and many of them are parasites of insects, plants or animals. They are structurally simple organisms and have been characterized as a tube within a tube; referring to the alimentary canal which extends from the mouth to tail. In size they range from 0.3 mm to over 8 meters.

The nematodes will occur around the plant root zone at depths 5-30cm or at the surface layers. Assessment of nematodes in fields can be done by taking individual samples or by taking a large number of samples from similar plants and bulking into one composite sample before extraction and further processing. The time of sampling can be critical. Number of nematodes will be lesser during the dry or winter months, outside the growing season. The most representative picture on nematodes will be obtained by sampling the plants from the middle to the end of the growing season.

Nematodes can be extracted from the soil by various techniques. Some of the methods are Baermann funnel method, tray method, Cotton wool filter method, flotation, sedimentation and sieving method, Cobb decanting and sieving method, elutriation, centrifugal flotation and flocculation and sieving method. Most extraction methods depend on either the Baermann funnel or its modifications where the motility of the nematodes is used to separate them from inert debris. Some of the common techniques are described here.

Baermann funnel techniques

This method requires a funnel with a piece of rubber tubing attached to the stem and closed by spring or screw clip. The funnel is placed in a support and almost filled with tap water. Finely crumbled soil is placed in a square of butter muslin which is folded to enclose the material, then gently submerged in the water in the funnel. Active nematodes pass through the cloth and sink to the bottom of the funnel stem. After some hours a small quantity of water containing the nematodes is run off and collected in a dish. The suspension from the dishes can be concentrated by passing it three or four times through 45 μ aperture sieve washing the nematodes off the sieve each time into a collecting vessel or through a bank of three or four such sieves.

Tray method

The basic tray method is where active nematodes move from the soil into dish of water by tissue or muslin cloth supported by various devices such as plastic sieves. Water is added to make the soil moist and the extraction left for 24hr after which the extraction filters is carefully removed and the water -nematode suspension poured off into beaker.

Sieving

Sieving involves pouring a suspension of soil (100-500ml in a bucket of water) through a bank of suitable sieves. Usually a sieve mesh of 75 μ m and 53 μ m are adequate and for maximum retention of nematodes ideally there should be two -three 53 μ m sieve in a bank with one or two 75 μ m sieves on top. The material trapped on each sieve is then washed into a beaker with a gentle jet of water applied to the back of each sieve.

Cotton wool filter method

Mix soil thoroughly in about 750 ml of water in a container and allow to stand for 10 sec. then decant into second container. Rinse remaining

soil in the first container 2-3 times and decant as above. Quickly pour off the whole suspension from the second container on to a double cotton wool filter about 18cm in diameter, supported on a coarse -mesh sieve followed by one liter of clean water. The suspension and the additional water should be poured on to a watch glass on the filter, which is just under water in a dish to prevent damage to the filter and get more even distribution. Then transfer the filter to a shallow extraction dish which contains just enough clean water to keep the filter wet. Carefully remove the filter after 12-24h when most of the active nematodes will have collected in the extraction dish.

Floataion sedimentation and sieving techniques

In this method soil sample is suspended in water and after a short period during which heavier particles are allowed to sink, the supernatant fluid is sieved. In general, most dorylaimids are caught on a 250 μ -aperture sieve, adults of average size nematodes on a 90 μ -aperture and many larvae and small adults on a 63 μ aperture sieve. A 45 μ aperture sieve is required for small larvae. Only a small portion of the nematodes are caught when a suspension is poured once through even the finest sieve. It is better to use a bank of sieves or to pour the suspension three or four times through the finest sieve in use, collecting the residue of the sieve each time. The diameter of the sieve, the quantity of water used and the amount of debris collected on the sieve will affect the number of nematodes retained.

Cobb decanting and sieving method

This method is used to get a general idea of the nematode fauna in a sample. Here a range of sieves is used through which a soil suspension is poured serially. Usually sieves having apertures 1mm, 710 μ , 250 μ , 150 μ , 90 μ and 63 μ are used.

Soak the soil sample for a few hours in a container. Stir the muddy mixture and pour it through a 1mm aperture sieve into a second container. Add more water and repeat the process. Rinse the material on the sieve either with a gentle jet of water so that the washings are collected in the second container or partially immerse the sieves and debris in the second container and gently shaking the sieve. The residue contains few larger nematodes.

After stirring the contents of second container pour them through 710 μ and repeat the procedure as above and collect the residue from the sieve. Repeat the operations with the remaining sieves, collecting the sievings each time in an appropriately labeled beaker.

Cleaning nematode suspension

Metal or plastic rings 6-8cm diameter and 2cm deep are fitted with a piece of butter muslin stretched over one end and held by a rubber band. A doubled piece of cotton wool milk filter or paper tissue is placed on this sieve and the suspension of nematodes and debris poured on to it. The nematodes are at first retained on the sieve which is then placed in clean water in a small dish until the debris is just immersed. This is left undisturbed for overnight; the nematodes wriggle through the filter and collect in the dish.

Killing and fixing

Nematodes are best killed by gentle heat (55-60°C) in water. After killing the sample is left to cool and then the whole nematodes suspension is fixed by adding an equal volume double strength fixative. Individual nematodes can also be picked and transferred to cold fixative at the normal strength.

Fixatives	Normal Strength	Double strength
1. TAF		
40% Formaldehyde (Formalin)	7ml	7ml
Triethanolamine	2ml	2ml
Distilled water	91 ml	45ml
2. FA 4:1		
40% Formaldehyde (Formalin)	10ml	10ml
Glacial acetic acid	1ml	1ml
Distilled water	89 ml	45ml
3. Formaldehyde (Formalin)	2% (5%)	4% (10%)

Nematodes in fixative should be left for 12h or overnight before processing

Storage of extract

Many nematodes remain in good condition for several days in water at 4°C. Contaminating micro organisms can be suppressed by adding 3 drops of 5% streptomycin sulphate solution at 5ml nematode suspension or by storing the nematodes in a 0.02% Aretan solution.

Conclusion

Several different techniques are applied to isolate and study microorganisms in pure culture. For isolation several different media are also employed. Among the various methods serial dilution agar plating method or viable plate count method is the most commonly used procedure for the isolation and enumeration fungi, bacteria and actinomycetes which are the most prevalent microorganisms. Similarly for nematode isolation, the Cobb decanting and sieving method and its modifications are used widely for the extraction of soil nematodes which is easy and less time consuming.

Selected references

- Aneja K R 2003 In: *Experiments in Microbiology Plant Pathology and Biotechnology* (pp157-188) New age International (P) Limited, Publishers, New Delhi
- Collins C H & Patricia M Lyne 1976 *Microbiological Methods* (4th edition), Butterworth & Co (Publishers) Ltd.
- Pelczar M J, Chan E C S & Noel R Krieg 1993 *Microbiology*. Tata Mc Graw-Hill Publishing Co Ltd., New Delhi
- Southey J F 1970 *Laboratory Methods for Work with Plant and Soil Nematodes*, Technical Bulletin 2, Her Majesty's Stationery Office. London
- Waller J M, Lenne J M & Waller S J 2002 *Plant Pathologists Pocket book*. CABI Publishing.

ANNEXURE 1

Nutrient agar

Peptone	5.0g
Beef Extract	3.0g
NaCl	5.0g
Distilled water	1000ml
pH	7.0

Glycerol yeast extract agar (pH 7.0)

Glycerol	5.0ml
Yeast Extract	2.0g

Dipotassium hydrogen phosphate	1.0g
Agar	15.0g
Aureomycin	0.01g
Distilled water	1000 ml

Potato dextrose agar (PDA)

Potatoes	200g
Dextrose	15g
Oxoid agar no:3	20g
Tap water	1000 ml

Czapek (dox) agar (CZ)

Sucrose (Analar)	30g
Agar	20g
Czapek stock solution A	50 ml
Czapek stock solution B	50 ml
Distilled water	900ml

Czapek stock solution A

Sodium nitrate (NaNO_3)	40g
Potassium chloride(KCl)	10g
Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	10g
Ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	0.2g
Distilled water	1000ml

Store in refrigerator

Czapek stock solution B

Dipotassium hydrogen phosphate(K_2HPO_4)	20g
Distilled water	1000ml

Store in refrigerator

Dissolve agar in distilled water using a double saucepan: add sucrose and stock solutions prior to autoclaving at 121°C for 20 min.

Sabouraud dextrose agar (SDA)

Dextrose (or maltose)	40g
Peptone	10g
Oxoid agar no:3	20g
Distilled water	1000ml

Dissolve agar, add dextrose and dissolve. Add peptone and adjust pH to 5.6. Autoclave at 121°C for 20 min.

***Trichoderma* specific media (Elad & Chet 1983)**

Magnesium sulphate	0.2g
Dipotassium hydrogen phosphate	0.9g
Potassium chloride	0.15g
Ammonium nitrate	1.00g
Glucose	3.00g
Chloramphenicol	0.25g
Ridomil	0.3g
PCNB	0.2g
Rose Bengal	0.15g
Agar	20g
Distilled water	1000ml

Rose bengal agar (Martin, 1950) (for fungi)

Potassium di hydrogen phosphate	1g
Magnesium sulphate	0.5g
Peptone	5g
Dextrose	10g
Rose Bengal	0.03g
Agar	20g
Streptomycin	1%
Distilled water	1000ml

Kings B medium (for fluorescent pseudomonas)

Proteose peptone	20.0g
K_2HPO_4	1.5g

Glycerol	10.0ml
MgSO ₄ ·7H ₂ O	1.5g
Agar	18g
Distilled water	1000ml
Cycloheximide	100µg/ml
Chloramphenicol	13µg/ml
Ampicillin	40µg/ml

N-free Bromothymol medium (for *Azospirillum*)

Malic acid	5.0g
KOH	4.0g
K ₂ HPO ₄	0.5g
FeSO ₄ ·H ₂ O	0.05g
MnSO ₄ ·H ₂ O	0.01g
MgSO ₄ ·7H ₂ O	0.1g
NaCl	0.02g
CaCl ₂	0.01g
Na ₂ MoO ₄	0.002g
Bromothymol Blue	2ml
Agar	17.5g
Distilled water	1000ml
pH	6.6-7.0

Pikovskayas agar (for phosphate solubilizing bacteria)

Glucose	10g
Ca ₃ (PO ₄) ₂	5g
(NH ₄) ₂ SO ₄	0.5g
KCl	0.2g
MgSO ₄ ·7H ₂ O	0.1g
MnSO ₄	trace
FeSO ₄	trace
Yeast Extract	0.5g
Agar	15g
Distilled water	1000ml

P₁₀ VP medium (for *Pythium* isolation)

Medium	Difco corn meal	:	17 g/L
Ingredients :	agar (CMA)		
	Pimaricin	:	10 ppm (= 10 ug/ml or 10 mg/ L
	Vancomycin HCL	:	200 ppm
	Pentachloronitro- benzene (PCNB)	:	100 ppm

Chemical	Final concentration In medium	Amount needed in IIX stock	
		Per ml	per 100ml
Pimaricin	Act. Ingr., 10 ug/ml	(110 ug/ ml)	
	If purity is 25 mg/ml. (2.5% suspension), use 0.0004 ml/ml.	0.0044 ml/ ml	0.44 ml/ 100 ml
	If purity is 90.5% (dry Powder), use 11.05 ug/ml.	121.55 ug/ ml	
Vancomycin	Act. Ingr., 200 ug/ ml	(2,200 ug/ ml)	
	If purity is 98%, use 204.1 ug/ ml.	2,245 ug/ ml	224.5 mg/ 100 ml
	If purity is 100%, use 200 ug/ ml.	2,200 ug/ ml	220 mg/ 100 ml
PCNB	Act. Ingr., 100 ug/ml	(1,100 ug/ml)	
	If purity is 75% (wett- able powder) use 133 ug/ml.	1,463 ug/ ml	146.3 mg/ 100 ml

- 1) If powdered Pimaricin is used, first dissolve the 12.2 mg of Pimaricin in 5 ml of dimethylsulfoxide (DMSO) in a sterile bottle, then add 95 ml of sterile distilled water. To the 100 ml of microsuspension of Pimaricin, add the powders of vancomycin and PCNB. The final concentration of DMSO in the P10VP medium is less than 0.5 %.
- 2) Add the IIX stock solution in an appropriate amount (10% of the volume of CMA) to the melted CMA temperature of about 43-45 °C.
e.g., 20ml of IIX stock to 200 ml CMA, making the total volume 220

ml or 50ml of IIX stock to 500 ml CMA, making the total volume 550 ml.

- 3) Mix the medium in the bottle by reciprocal tilting action (Do not mix by shaking).
- 4) When used in soil dilution plate experiments, pour 15-16 ml medium into each 100-mm Petri plate top which 1 ml soil suspension has been added earlier.
- 5) Incubate the plates in the dark.

PVPH medium (for *Phytophthora* isolation)

Medium	Difco corn meal	: 17 g/L
Ingredients :	agar (CMA)	
	Pimaricin	: 10 ppm (= 10 ug/ml or 10 mg/L
	Vancomycin HCL	: 200 ppm
	Pentachloronitro- benzene (PCNB)	: 100 ppm
	Hymexazol	: 50 ppm

Chemical	Final concentration In medium	Amount needed in IIX stock	
		Per ml	per 100ml
Pimaricin	Act. Ingr. 10 ug/ml	(110 ug/ ml)	
	If purity is 50% use 20 ug/ ml	220 ug/ ml	22 mg/100ml
	If purity is 90.5%, use 11.05 ug/ml.	121.55 ug/ ml	12.2 mg/100ml
Vancomycin OR	Act. Ingr. 200 ug/ ml	(2,200 ug/ ml)	
	126 mg/ 100 ml of Ampicillin	If purity is 98%, use 204.1 ug/ ml.	2,245 ug/ ml 224.5 mg/ 100 ml
	If purity is 100%, use 200 ug/ ml.	2,200 ug/ ml	220 mg/ 100 ml
PCNB 75%	Act. Ingr. 100 ug/ml	(1,100 ug/ml)	

	If purity is 75%, use 133 ug/ml.	1, 463 ug/ ml	146.3 mg/ 100 ml
Hymexazol	Active ingredient, 50 ug/ml	(550 ug/ml)	
	If purity is 99.4%, Use 50.3 ug/ ml	553 ug/ ml	55.3 mg/ 100 ml

1. To prepare 100ml of IIX stock solution, first dissolve the Pimaricin powder in 5 ml of dimethylsulfoxide (DMSO) in a sterile bottle, then add 95 ml of sterile distilled water. (For 150ml of IIX stock, for example, use 7.5 ml of DMSO). To the 100 ml of microsuspension of Pimaricin, add the (powders of Vancomycin, PCNB, and Hymexazol. The final concentration of DMSO in the PVPH medium is less than 0.5%.
2. Add the IIX stock solution in an appropriate amount (10% of the volume of CMA) to the melted CMA at temperature of about 43-45°C.
3. Mix the medium in the bottle by reciprocal tilting action. (Do not mix by shaking)
4. When used in soil dilution plating, pour 15-16ml medium into each 100 mm Petri plate to which 1 ml soil suspension has been added earlier.
5. Incubate the plates in the dark.

4

Preparation of reagents and DNA isolations

A Kumar, R Aravind & V Vinod

1. Basic techniques: Agarose gel

Reagents and material

Agarose

Tris (hydroxymethyl)-amino methane.

Na-EDTA.

Boric acid.

Ethidium bromide

(1,7-diamino-10-ethyl-9-phenylphenanthridiniumbromide)

Sodium hydroxide pellets, NaOH

TAE 50x.

Solutions

0.5M EDTA: Add 186.1 g Na-EDTA to 800 ml H₂O and stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with NaOH. Autoclave the solution. EDTA will dissolve at pH 8.0.

TBE-buffer 5x stock: Add 54 g Tris and 27.5 g boric acid to 800 ml ultra pure H₂O. Add 20 ml 0.5M EDTA. Adjust volume to 1000 ml with ultra pure H₂O.

TBE-buffer 0.5x: Before use the TBE-buffer 5x has to be diluted 10x: mix 100 ml TBE-buffer 5x with 900 ml ultra pure water.

Ethidium bromide: Dissolve 10 mg ethidium bromide in 10 ml ultra pure H₂O.

TAE-buffer 1x: Mix 200 ml TAE 50x with 9800 ml ultra pure water.

Protocol

- Transfer the appropriate amount of agarose to a 300 ml Erlenmeyer flask.
- Add half of the total amount of buffer, but at least 100 ml to the agarose.
- Heat the solution in a microwave oven; at high concentrations agarose will start to foam, stop the cooking process before it boils over.
- Rotate the Erlenmeyer flask carefully.

- Leave the Erlenmeyer-flask for a few minutes and repeat step 3 for 10-20 seconds, until agarose has dissolved completely.
- After dissolving, boil agarose for 10-15 seconds.
- Adjust volume with the rest of buffer to the right volume. Mix warm and cold solutions. Heat solution in microwave oven if necessary.
- Leave agarose to cool to 55-60°C.
- Pipette ethidium bromide and swirl Erlenmeyer-flask.
- Tape gel tray on both sides and water level it.
- Pour agarose solution into the gel tray after cooling (55-60°C).
- Swirl Erlenmeyer flask occasionally.
- Remove air bubbles with a pipette tip and place the comb(s).
- Leave agarose to solidify. After solidifying, the agarose can be cooled in a refrigerator.
- Remove tapes and place gel tray into the electrophoresis-container; the electrophoresis-container is filled with the same buffer as used for the agarose gel.
- Remove comb(s) carefully without damaging the slots.
- Add a sufficient amount of buffer to the electrophoresis-container to cover the gel with a thin layer of buffer.

Remarks

At high concentration, TBE may precipitate after long-time storage, showing a white layer. To prevent this, 5x solutions of TBE should be kept at room temperature in glass bottles. If the buffer shows precipitation, it should be thrown away. In the original protocol a 1x buffer solutions is used for agarose gel electrophoresis, but a 0.5x solution has enough buffer capacity though. For TAE-buffer the concentration should be 1x to have enough buffer capacity.

Ethidium bromide is a potent carcinogen and mutagen. Literature is not always clear about it, but it is advised to wear protective clothes while handling. Use goggles and gloves. By influence of UV-light ethidium bromide becomes less mutagenic/ carcinogenic. Solutions and gels containing ethidium bromide should be decontaminated before they are disposed of.

The level of buffer above the gel should be as small as possible. With a high level of buffer the current will not entirely go through the gel but also through the buffer.

2. General methodology for isolation of DNA from fungi and other microorganisms

Fungal cells contain a thick rigid cell wall. When isolating DNA from fungus, the yield and quality of DNA depends on the extent of rupturing cell wall and the degree of carbohydrate and protein contamination. The best protocol is the one that effectively ruptures the cell wall and minimizes contamination.

CTAB method

One gram of four days old fungal mycelium is ground in liquid N₂ and immediately transferred to 5ml CTAB extraction buffer (2%w/v CTAB, 100mM Tris-Cl, 20mM EDTA pH 8.0, 1.4M NaCl) preheated at 65°C. The contents are mixed well and incubated at 65°C for 30 minutes. DNA is extracted with equal volume of chloroform: isoamyl alcohol (24:1) for 15 minutes on a rocking platform. The two phases are separated by centrifugation at 12,000 rpm for 5 minutes. The aqueous phase is separated and the DNA is precipitated with 2/3rd volume of isopropanol. DNA is pelleted by centrifuging at 12,000 rpm for 5 minutes. The pellet is air dried and dissolved in 500ml of sterile distilled water.

SDS method

One gram of mycelium is ground using liquid N₂ and transferred to a sterile centrifuge tube. To this 10ml of STE extraction buffer (320mM Sucrose, 10mM Tris-Cl, 20mM EDTA, 75mM NaCl and 2.5ml of 20% SDS) is added and incubated for 10 minutes in water bath followed by extraction with equal volume of phenol: chloroform (1:1). The aqueous phase is taken and DNA precipitated with 2/3rd volume of isopropanol. DNA is pelleted by centrifugation at 14,000 rpm for 15 minutes and washed with 70% ethanol, air-dried and dissolved in 500ml of sterile distilled water.

SDS method using powdered glass

In this method, fine powdered glass made from glass wool is employed. One gram of mycelium is ground with powdered glass with enough STE buffer to saturate the powdered glass. The rest of the method is same as in the above protocol.

SDS method using minigrinder

This method is the most successful one giving good quality DNA in high yield. To effect disruption of rigid chitinous cell wall, a minigrinder (Sigma) is employed. This minigrinder is designed such that it fits in the microfuge tube and effectively disrupts the cell wall which is reflected in the high DNA yield.

DNA isolation from *Phytophthora*

0.1g of four days old mycelium + 750ml of SDS extraction buffer + 50mg of polyvinyl polypyrrolidone + 10mg of glass powder

Grind well with minigrinder.

Centrifuge at 13,000 rpm for 5 minutes.

Add mixture of phenol: chloroform: isoamyl alcohol (25:24:1) to supernatant
Mix well.

Centrifuge at 13,000 rpm for 5 minutes.

Add isopropanol (2/3 vol).

Centrifuge at 13,000 rpm for 10 minutes.

Look for DNA pellet and wash the pellet in 70% ethanol by centrifuging at 13,000 rpm for 2 minutes.

Dry the pellet and add 50ml of sterile distilled water with RNase (20ug/ml).

Isolation of DNA from *Trichoderma*

Trichoderma source

The *Trichoderma* isolates can be obtained from wide variety of agroecosystems. Many of them have been tested as effective biocontrol agents against a number of plant pathogens. Some of them have remarkable resistance against metals and a few have very good commercial prospects.

Purification of *Trichoderma*

With the help of a sterile loop, fungal spores were taken from agar slants and resuspended in 1ml sterile distilled water in a microfuge tube. The suspension was serially diluted to 10^{-4} dilution. Isolated colonies were obtained by pour plating and/or by streaking on *Trichoderma* specific medium (TSM). The plates were incubated at 28°C.

Production of fungal mycelium

For submerged culture, well-isolated colonies are used for inoculation in 50 ml of TSM basal broth and the fungus is cultivated with constant shaking at 180 rpm and 28°C.

Preparation of mycelium for DNA isolation

Four days old submerged culture mycelium is used for DNA isolation. The mycelium is filtered out on a sterile Whatman No. 1 filter paper and washed thrice with sterile distilled water to remove traces of the medium. The fungal mass is air dried to remove excess of moisture. Mycelium is used either fresh or stored at -80°C for subsequent use.

DNA isolation from fungi by SDS method (eg. *Trichoderma harzianum*)

Transfer 200mg mycelium in a microfuge tube.

Add 100ml STE extraction buffer and grind well for 3 minutes using minigrinder.

Add 400ml STE buffer and grind again for 2 minutes.

Finally add 500ml of STE buffer and 250ml of 20% SDS.

Mix well and incubate at 65°C for 10 minutes.

After incubation divide the contents of the tube into two microfuge tubes.

Add equal volume of phenol: chloroform (1:1) and mix gently.

Centrifuge at 5000 rpm for 5 minutes to separate the phases.

Re-extract aqueous phase with phenol: chloroform (1:1).

Collect the aqueous phase and precipitate DNA with 1/10th volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol.

Wash the pellet with 500ml of 70% ethanol.

Dry pellet in vacuum for 20 minutes and dissolve in 100ml sterile distilled water.

Store at 4°C.

DNA isolation from nematodes

Incubate nematode suspension in 100 ml of extraction buffer (100mM Tris-Cl pH 8, 100mM EDTA, 100mM NaCl, 0.5% SDS and 200 mg of proteinase K) at 55°C for 3 h.

Grind the nematode suspension using a micro homogeniser to facilitate lysis before incubation.

Dilute the samples with buffer saturated phenol and extract the DNA with chloroform- isoamyl alcohol (24:1).

Precipitate the DNA with isopropanol and dissolve in TE buffer (10mM Tris-Cl, 0.1mM EDTA pH 8).

Quantify the DNA in a biophotometer.

Isolation of DNA from bacteria

Chemicals and reagents

Extraction buffer: 100 mM Tris HCl; 100 mM EDTA; 100 mM Na₂ HPO₄; 1.5 mM NaCl; 1% CTAB; Proteinase K-0.03µg/ml; Lysozyme -0.67µg/ml; (Proteinase K and Lysozyme are heat labile. So these are added at the time of use); SDS 20%; Chloroform: isoamyl alcohol (24:1); Isopropanol; Ethyl alcohol 70%.

Protocol

One milliliter of bacterial culture (OD 0.5-1.0 at 600nm), Centrifuge at 10000G 20 min at 4°C.

Resuspend the pellet with sterile distilled water. Centrifuge at 10000G 20 min at 4°C. Clean the pellet two times with sterile distilled water. Add 675 µl of extraction buffer and incubate at 37°C for 30 min. Add 75µl of SDS (20%). Incubate at 65 °C for 2 hours. Centrifuge at 6000G for 10 min at 4°C. Collect the clear solution in a sterile Eppendorf tube. Add equal volumes of Chloroform :Isoamyl alcohol (24:1). Centrifuge at 10000 G for 10 min at 4°C. Remove the aqueous phase to a sterile Eppendorf tube. Add 0.6 volumes of isopropyl alcohol and incubate at room **temperature** for 1 hour. Centrifuge at 10000 G for 10 min. Wash the pellet in 500µl of 70% ethanol. Centrifuge at 10000 G for 10 min at room **temperature**. Dry the pellet and dissolve in sterile distilled water.

3. Quality Analysis of DNA in agarose gels

The quality of isolated fungal genomic DNA is checked in 0.7% agarose gel.

Melt 0.28g of Agarose in 40 ml of 1x TAE in a 150 ml flask by heating the flask in a microwave oven.

Cool the molten agarose to 50°C and add 2ml of EtBr (10mg/ml). Cast a gel in a gel-casting tray with properly placed combs.

Mix 10ml of DNA and 2ml of gel loading buffer and load the mix to each well without spillover.

Subject the gel to 3V/cm electricity for 1 hour in 1X TAE buffer.

Visualize the gel in Multi-image light cabinet (Alpha Imager 2200) and the gel images can be captured by Charge-Coupled Device (CCD) camera. Store the images as JPEG or TIFF extension file for analysis.

Dilute all the DNA samples ten fold times and then quantify in a UV-VIS spectrophotometer. The A260/A280 ratio is calculated to check the purity of the DNA preparation.

Note: Visit www.molbiol.ru for complete details of reagent preparation in the day-to-day molecular biology experiments.

5

Purification of plant viruses

A Ishwara Bhat

Isolation and purification of plant viruses are performed to separate virus particles from plant constituents. Purified virus preparation is important to study basic properties of a virus. The objective of purification is to produce preparation containing only infective virus particles although it is difficult to remove the last traces of host constituents. But plants infected with some viruses contain a variable of virus products (particles of different length and composition), and some virus particles lose their infectivity during purification.

The first step in the virus purification is to crush the cells and extract the virus containing sap. Most of the larger constituents of sap such as chloroplasts, mitochondria, starch grains, fragments of cell walls sediment quickly and can be removed by brief low g centrifugation. Sap constituents such as plant proteins, ribosomes and microsomes resemble virus particles in size, composition and stability and that are the most troublesome host materials to remove when purifying viruses.

Stages in purification

Propagation of virus

Selection of the right kind of host plant for propagation of virus to be purified is of very important. A good propagation host should be susceptible and show high concentration in the plant, should not contain high amount of phenolic compounds, mucilage, latex or other inhibitors. Some of the plant species in the genera, *Chenopodium*, *Cucumis*, *Nicotiana*, *Petunia* and *Vigna* have been found to be suitable propagation hosts for purification of large number of viruses. The conditions under which the plants to be grown and the time at which it is harvested should be standardized to maximize the starting concentration of the virus.

Extraction of virus

Many viruses require a particular pH and ion strength for their stability. Buffer solutions with pH values in the range of 7-8 are generally used during extraction to keep the virus particles in suspension. Some of the commonly used buffers include: borate, citrate, phosphate and tris.

Oxidation affects stability of a virus during extraction, often leading to loss of infectivity. Hence reducing agents such as sodium sulfite, sodium thioglycolate, 2 mercaptoethanol, cystine hydrochloride are added to the extraction buffer. Additives such as EDTA (sodium salt) are added to break down ribosomes. However, this can be used only for viruses that do not require divalent metal ions for stability.

The particles of many viruses aggregate and many compounds have been used to reduce this tendency. Detergents such as Triton X 100, Tween 80 have been used. For potyviruses, use of urea was found useful in reducing aggregation. Use of enzymes like pectinase and cellulase aids in both release of virus particles that would otherwise remain in the fiber fraction. A variety of procedures are used to crush or homogenize the virus infected tissue like pestle and mortar, blenders and juice extractors, roller mills or meat mincer. The crushed tissue is usually expressed through muslin cloth.

Clarification of the extract

The first step in virus purification is to remove macromolecular host materials, leaving the virus in solution. This can be achieved by addition of organic solvents, heating or addition of dipotassium hydrogen. The treated extract is then subjected to centrifugation at low speed (10,000g for 10-20 min).

Concentration of the virus

From the clarified extract, the virus can be concentrated by any of the following methods like centrifugation, precipitation with polyethylene glycol, precipitation with salt or precipitation at isoelectric point.

Further of the virus

Even after concentration, virus preparations still contain some low and high molecular weight host materials. Most of them can be removed by further purification. Further purification can be achieved by differential centrifugation (alternate cycles of high and low centrifugation), density gradient centrifugation, gel filtration or affinity column bound with specific antibodies.

Purification protocol for *Cucumber mosaic virus* (CMV)

Materials required

Equipments: pH meter, magnetic stirrer, balance, waring blender, high speed refrigerated centrifuge, Ultracentrifuge, refrigerator, autoclave, ice flaking machine

Glassware/Plasticware: Beakers, conical flasks, reagent bottles, measuring cylinder, eppendorf tubes, pipettes, microtips, centrifuge tubes, Pasteur pipette

Consumables: Distilled water, sodium acetate buffer, EDTA, thioglycolic acid, chloroform, polyethylene glycol, borate buffer, sucrose, Triton X 100

Protocol

1. Homogenise the leaves in a blender in extraction buffer (0.05M sodium acetate buffer, pH 6.4) containing 5 mM ethylene diamine tetra acetic acid (EDTA) di-sodium salt and 0.5% thioglycolic acid.
2. Filter the extract through muslin cloth and emulsify with an equal volume of chloroform by stirring for 30 min at 4°C.
3. Centrifuge the extract at 12000g for 10 min.
4. Discard the pellet and add to the supernatant polyethylenc glycol (PEG 8000, 10%) in presence of sodium chloride (1.75% w/v) with continuous stirring for 1 h.
5. Centrifuge the mixture at 12,000g for 15 min.
6. Discard the supernatant and resuspend the pellet overnight in one-fifth volume of 0.5M sodium acetate buffer containing 5mM EDTA, pH 9.0.
7. Add Triton X 100 to a final concentration of 2% (v/v) and stirr for 30 min
8. Centrifuge at 18,000g for 25 min.
9. Discard the pellet and the supernatant subjected to centrifugation at 45,000 g for 3h.
10. Discard the supernatant and suspend the pellet in 5 mM borate buffer containing 0.5 mM EDTA, pH 9.0.
11. Further purification is carried out by layering partially purified preparation on a linear 10-40% (w/v) pre-formed sucrose density gradient (prepared by layering 5 ml of 10% and 10 ml each of 20%, 30% and 40% sucrose at 4°C).
12. Centrifuge the gradients at 55,000g for 2 h using swing out rotor (SW 28, Beckman).
13. After the run, remove the gradient tube and locate the virus containing band with the help of light vertically passing through the tube.
14. Collect the virus band using either a syringe or a Pasteur pipette, or a fraction collector.

15. Dilute the virus preparation with resuspension buffer and pellet the virus by centrifugation at 45,000g for 3 h.
16. Discard the supernatant and resuspend the pellet in a small volume of re-suspension buffer.

Selected references

Mossop D W, Francki R I B & Grivell C J 1976 *Virology* 74: 544-546.

Moghal S M & Francki R I B 1976 *Virology* 73: 350-362.

Hull R 2003 In: *Matthew's Plant Virology*, Academic Press, New York.

6

Enzyme linked immunosorbent assay (ELISA)

A Ishwara Bhat

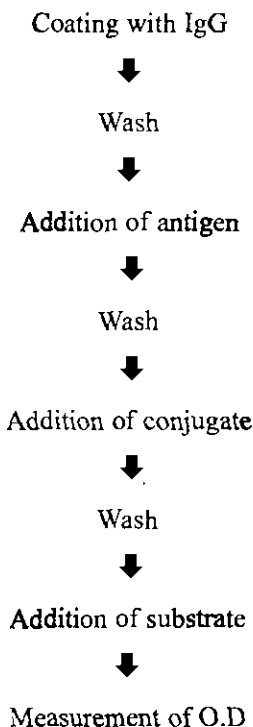
The reaction between antibodies and antigen paved the way for the detection of plant pathogens especially viruses and these techniques are referred to as serological techniques. A variety of methods have been developed for demonstrating and estimating combination between antibodies and antigens. The traditional methods for using antisera with plant viruses involved direct observation of specific precipitates of virus and antibody, either in liquid media or in agar gels. Over about the past 10 years these methods have been progressively superseded by the use of enzyme-linked immunosorbent assay (ELISA). ELISA is one of the most widely used serological tests for the detection of plant viruses because of its simplicity, adaptability and sensitivity. In this the specificity of antigen-antibody reaction is recognized through the action of the associated enzyme label on a suitable substrate unlike observing the formation of an insoluble antigen-antibody complex in precipitin reactions. This method is very economical in the use of reactants and can be applied to viruses of various morphology both in purified and crude preparations. Method is very useful when large number of samples is to be tested and is very sensitive in detecting up to 1-10 ng per ml of virus. Sensitivity of detection of antibody-antigen reaction is increased by attaching antibody to a minute quantity of enzyme. As enzyme substrate is then added, and the resulting colour reaction may be quantitatively measured.

Two forms of ELISA are used – (i) direct ELISA wherein the virus (antigen) is trapped by antiviral antibody and detected using enzyme labeled virus specific antibody, (ii) In the indirect ELISA, the enzyme used in the final detection and assay step is conjugated to an antiglobulin antibody. (For example- if the virus antibodies were raised in rabbit, a chicken/horse/elephant/goat anti-rabbit globulin might be used).

One of the commonly used techniques of direct ELISA is double antibody sandwich ELISA (DAS-ELISA) while direct antigen coated ELISA (DAC-ELISA), $F(ab')_2$ ELISA and triple antibody sandwich ELISA (TAS-ELISA) are some techniques under indirect ELISA.

Direct Double Antibody Sandwich ELISA (DAS-ELISA)

In this procedure, wells of polystyrene plate are first coated with IgG. The test sample of virus is then added to the adsorbed antibody followed by addition of enzyme labeled antibody to the trapped virus. The attached enzyme subsequently digests an added enzyme substrate which results in a colour development. The procedure requires isolation of immunoglobulins (IgG) from crude antiserum and preparation of immunoglobulin – enzyme conjugate. The intensity of the colour is directly proportional to the amount of virus. Use of proper negative, buffer and positive controls are important while performing ELISA.



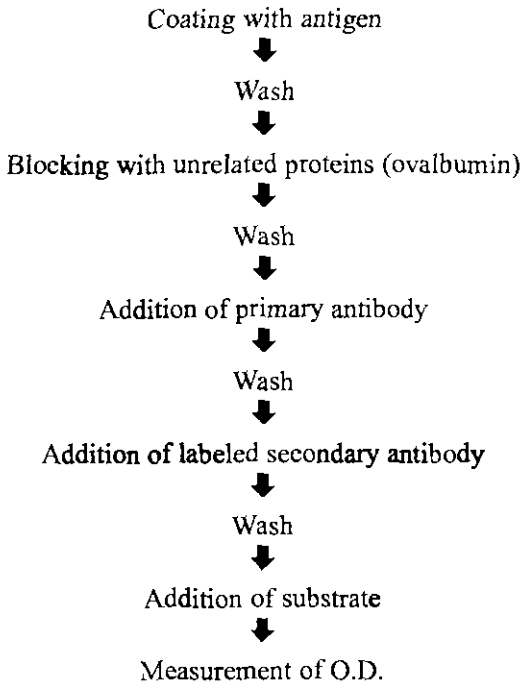
Schematic flow chart showing steps in DAS-ELISA

Direct Antigen Coated ELISA (DAC-ELISA)

DAS-ELISA requires preparation of conjugates specific to each of the viruses to be tested thus making it cumbersome and expensive. This problem is overcome in DAC-ELISA. In DAC-ELISA, the presence of the antigen-primary antibody complex is detected by antibody produced against the immunoglobulin fraction of blood of animal species from which the

primary antibodies had been obtained. These antibodies are called as secondary antibodies. Secondary antibodies are labeled with enzyme and used in detection. These conjugates are called as 'universal conjugates' as they can be used for detecting all viruses against which primary antibody are produced in one kind of animal (such as rabbit). Commonly used conjugates of this type are alkaline phosphatase labeled goat anti-rabbit IgG (for polyclonal antibodies as many polyclonal antibodies are produced in rabbits) or alkaline phosphatase labeled goat anti-mouse IgG (for monoclonal antibodies). The sensitivity of DAC-ELISA is usually lower than that of DAS-ELISA. However, it is better suited to study serological relationships among different viruses.

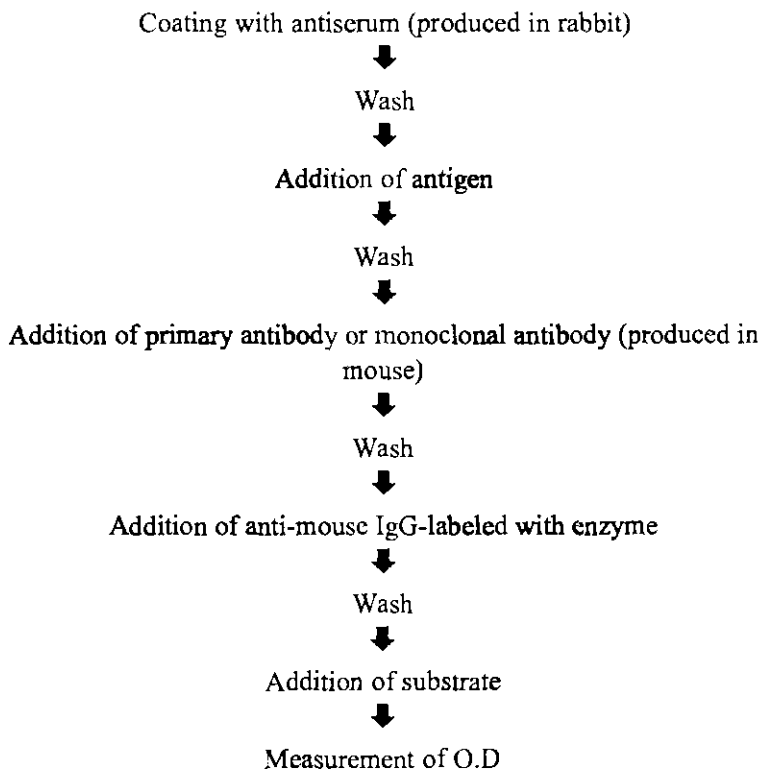
The important steps in DAC-ELISA include: coating the wells of microtitre plate with antigens, followed by blocking the remaining reactive sites of the wells by addition of non-specific heterologous proteins such as bovine serum albumin or non fat spray dried milk. Wells are then added with primary antibody (not labeled with enzyme). The detection of the reaction is done by addition of enzyme labeled second animal antibody (against antibodies of the first animal) and its substrate.



Schematic flow chart showing steps in DAC-ELISA

Plate trapped or triple sandwich ELISA

This is a form of indirect ELISA wherein antibody produced in two different animal systems against the same virus is used. The method is routinely used in monoclonal antibody production for screening of hybridoma. Initially, polyclonal antiserum produced in rabbit is used to trap the antigen. After addition of antigen, the hybridoma secreting antibodies (obtained by fusing spleen cells of mouse injected with the virus and cancerous cells) are used as detecting antibody. The antigen-antibody reaction is visualized by adding anti-mouse IgG- alkaline phosphatase.



Schematic flow chart of triple sandwich or plate trapped ELISA

Dot immunobinding assay (DIBA) or dot ELISA

In this procedure instead of microtitre plate, nitrocellulose membrane (NCM) is used as solid phase. Plant extract is dotted onto NCM directly or on a pre-coated NCM (with antibodies). For the final colour development, a substrate is added that the enzyme linked to IgG converts into an insoluble material. Intensity of colour can be read visually or through densitometer.

Protocol for DAS-ELISA utilizing alkaline phosphatase

Requirements

Materials:

Equipments: Incubator, magnetic stirrer, pH meter, ELISA reader, Microtitre (ELISA) plate, Micropipette and tips: Use adjustable volume micropipette

Multichannel pipette (adjustable 100 to 300 μ l) (Optional): Multichannel pipettes which can hold four, eight or 12 microtips thus permitting dispensing of volumes simultaneously into several wells can be used to add antigen, antiserum and conjugates.

Glassware/plasticware: Beakers, measuring cylinders, pipette, microtips, pestle and mortar.

Reagents:

IgG (against virus of interest)

Coating buffer (Carbonate buffer): Dissolve 1.59g Na_2CO_3 , 2.93g NaHCO_3 , and 0.20g NaN_3 in one litre of distilled water.

Phosphate buffered saline (PBS) : Dissolves.0g NaCl, 0.2g KH_2PO_4 , 2.9g Na_2HPO_4 , 0.2g KCl in distilled water and make up the volume to 1000 ml.

PBS-T: Add 0.05% Tween 20 in 1X PBS

Antigen extraction /antibody buffer: Add 2% polyvinyl pyrrolidone (PVP, MW 40,000) and 0.2% albumin in PBS-T.

IgG-alkaline phosphatase conjugate

Substrate buffer: Dissolve 97 ml diethanolamine in 800 ml distilled water, adjust pH to 9.8 with concentrated HCL, make up the volume using distilled to 1000 ml.

Para nitrophenyl phosphate (PNPP): Commercial preparation available either in powder or tablet forms may be used. Protect from light while storing.

Protocol

1. Add 200 μ l of IgG diluted in coating buffer to each well of microtitre plate and incubate at 37°C for 2 h.
2. Well contents are discarded and washed with PBS-T (flooding with three changes of PBS-T for three min each time). After final washing, plates are shaken dry over paper towel.
3. Test samples are extracted in antigen extraction buffer in 1:10 (w/v) using pestle and mortar. Extracts of healthy plants and the extraction buffer are to be used as negative control, whereas, extracts from known infected plant should be used as positive control. Aliquots (200 μ l) of test sample are added and plates incubated overnight at 4°C.

4. The plates are washed as mentioned in (2) and diluted enzyme conjugate (200 μ l per well) in antibody buffer is added and incubated at 37°C for 2 h.
5. After washing as described in (2), 200 μ l of substrate (p-nitro phenyl phosphate 0.6 mg/ml of substrate buffer) is added to each well and incubated at room temperature to develop colour.
6. Results are recorded by measuring the absorbance at 405nm in an ELISA reader after 30, 60 and 120 min after substrate addition.
7. Stop the reaction if required by adding 100 μ l of 3 M NaOH solution to each well.

Selected references

Avrameas S 1969 *Immunochemistry* 5 : 43-52.

Barbara D J & Clark M F 1982 *J. Gen. Virol.* 58 : 315-322.

Clark M F & Adams A N 1977 *J. Gen. Virol.* 34 : 475-483.

Clark M F, Lister R M & Bar-Joseph M 1986 *Methods Enzymol.* 118 : 742-766.

Hampton R, Ball E & De Boer S 1990 In: *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens*, APS Press, pp. 389.

Koenig R 1981 *J. Gen. Virol.* 55 : 53-62.

Koenig R & Paul H L 1982 *J. Virol. Methods* 5 : 113-125.

7

Cloning of PCR product

A Ishwara Bhat

The validity of the amplification reaction can be assessed by cloning product followed by sequencing. There are three basic methods in use for cloning a PCR product. They include: (i) TA cloning: Since the PCR product generated by *Taq* polymerase is appended with a single extraneous dA at 3' ends, the easiest way of cloning is by using plasmid tailed with dT. (ii) Blunt end cloning: The blunt ended PCR product generated by *Pwo* or *Pfu* polymerase can be cloned into a plasmid restricted with blunt end generating enzymes. (iii) Directional cohesive end cloning: In this case PCR product is first restricted with appropriate restriction enzymes followed by ligating them onto plasmid linearized by same restriction enzymes. In all the above methods PCR products are first purified to remove enzymes, unused primers, dNTPs etc. For this the PCR product is first run on low melting agarose gel, the band of interest is excised and purified. Alternatively PCR product can also be purified using commercial kits. The purified PCR product is then ligated with linearized dT or blunt end or cohesive end vector using *E. coli* T₄ DNA ligase. If directional cloning is planned, restriction of PCR product is carried out with appropriate enzymes before ligation with vector. The *ligated vector is then used to transform competent E. coli cells and transformants are selected using appropriate markers such as antibiotic or blue / white colony morphology.* The following protocol describes cloning using TA vector.

Protocol

1. Prepare 0.8% LMP agarose in 1x TAE buffer and cool it to room temperature. Cast the gel on a pre-cooled gel set and allow it to solidify at 4°C.
2. Load PCR product with loading dye and run at 100 V for 60-90 min.
3. Examine the gel under UV transilluminator to locate the DNA band of interest. Excise the band using a clean sterile razor blade or scalpel.
4. Trim off any excess agarose and transfer to a 1.5 ml sterile eppendorf tube.

5. Incubate the tube at 70°C in a heating block for 7 min.
6. Cool the solution to room temperature; add equal volume of buffer saturated phenol and vortex for 30 sec.
7. Centrifuge for 2 min at 10,000 rpm and collect aqueous phase.
8. Re-extract the aqueous phase once with phenol: chloroform and then twice with chloroform alone.
9. Precipitate the DNA by adding 0.1 volume of 3M chilled sodium acetate and 2 volumes of ethanol. Incubate the mixture at -80°C for 1h.
10. Centrifuge for 15 min at 12,000 rpm at 4°C to pelletize the DNA.
11. Pour off the supernatant carefully and wash the pellet with 70% cold ethanol and re-centrifuge for 5 min.
12. Pour off the supernatant carefully, air dry the pellet and resuspend the pellet in a small volume (about 10-20 μ l) of sterile water and store at -20°C until ready to use.

Purification of PCR product through low melting point (LMP) agarose gel

Requirements

<i>Materials</i>	<i>Reagents</i>
Balance	Buffer saturated phenol
Beakers, measuring cylinders	Chloroform
Microwave oven	Phenol: chloroform
Low melting point (LMP) agarose gel apparatus and power pack	3M sodium acetate pH 5.2
Microcentrifuge tubes, microtips	Absolute and 70% ethanol
pH meter, magnetic stirrer	Sterile water
Transilluminator	
Waterbath	
Microcentrifuge	
Micropipette	
Razor blade	
Refrigerator/cold room	

Ligation of the purified PCR product into vector

Requirements

Materials	Reagents
Refrigerator	10x T4 DNA ligase buffer
Micropipette and tips	T4 DNA ligase
Microcentrifuge	Linearized TA vector
	Purified PCR product

Protocol

1. Set up ligation reaction with purified PCR product in a TA plasmid vector.
10x T4 DNA ligase buffer 1.0 μ l
Linearized TA vector (20 ng/ μ l) 1.0 μ l
Purified PCR product (10 ng/ μ l) 7.0 μ l
T4 DNA ligase (3 Weiss units/ μ l) 1.0 μ l
2. Mix the reactants by pipetting and incubate at 4°C for 24 h.
3. Ligated plasmid is now ready for transformation.

Preparation of competent *E. coli* cells

Plasmid DNA do not enter bacteria under their own power, but require assistance traversing the outer and inner cell membranes and in reaching an intracellular site where they can be expressed and replicated. This can be achieved by using competent bacterial cells for transformation. Bacterial cells can be made competent (to take up foreign DNA) either by chemical or physical methods. Most of the chemical methods are based on the observations of Madel and Higa (1970) who showed that bacteria treated with ice cold solutions of CaCl_2 and then briefly heated to 37°C or 42°C could be transfected with bacteriophage lamda DNA. The same method was later used to transform bacteria with plasmid DNA. The physical method known as electroporation involve exposure of bacterial cells to electrical charge that destabilizes their membranes and thus induces the formation of transient membrane pores through which DNA molecules can pass. Although

electroporation is easy, fast and efficient, it is expensive requiring costly electrical equipment and specially designed cuvettes. In the following the commonly and most frequently used method of preparation and transformation of competent *E. coli* using CaCl_2 is described.

Requirements

Materials	Reagents
Ice flaking machine	<i>E. coli</i> bacterial strain (DH 5)
High speed centrifuge and centrifuge tubes	Luria broth and Luria agar
Autoclave	0.1M MgCl_2
Glasswares	0.1M CaCl_2
Micropipette and tips	
Shaker incubator	
Laminar flow	
Refrigerator	
Sterile Petri plates	
Inoculation needle	
Eppendorf tubes	

Protocol

1. Pick a single bacterial colony from a freshly prepared plate and transfer into 100 ml broth. Incubate the culture with vigorous shaking (200 rpm) at 37°C till the O.D. reaches 0.4 at 600 nm.
2. When the required O.D. is reached, transfer the bacterial cells to sterile disposable, ice cold 50 ml polypropylene tubes. Store on ice for 10 min.
3. Centrifuge the cells at 2700 g for 10 min at 4°C.
4. Decant off the supernatant and resuspend the pellet by swirling in 30 ml of ice cold MgCl_2 - CaCl_2 solution (80 mM MgCl_2 , 20 mM CaCl_2).
5. Recover the cells by centrifugation as above.
6. Decant the medium from pellets and resuspend the pellet in 2 ml of ice cold 0.1M CaCl_2 for each 50 ml of original culture.

7. At this point cells achieve competence. The cells can be directly used for transformation or can be dispersed into aliquots and frozen at -70°C for later use.

Transformation of *E. coli*

Requirements

<i>Materials</i>	<i>Reagents</i>
Microcentrifuge	Appropriate antibiotics solution
Eppendorf tubes	Competent cells
Micropipette and tips	Recombinant ligated plasmid
Laminar flow	X-gal
Incubator cum shaker	IPTG
Ice flakes	
Water bath	
Luria broth and Luria agar	
Spreader	

Protocol

1. Take 200 μl of competent cells in a sterile chilled tube. Add about 50 ng recombinant plasmid (ligation mix) in a volume of 10 μl or less. Also, keep a control for transformation, by incubating 2 ng of plasmid with 200 μl of competent cells. Have one extra tube of cells as untransformed control to check for contamination.
2. Mix gently and incubate on ice for 30- 45 min.
3. Transfer the tubes to 42°C in a water bath for 90-120 sec (to give heat shock to cells).
4. Quickly transfer tubes to an ice bath and chill for 1-2 min.
5. Add 1 ml of LB medium and incubate at 37°C for 1 h with shaking (at 200 rpm).
6. Plate the cells on appropriate selective medium (Luria agar containing x-gal, IPTG and appropriate antibiotics) using different volumes (200 μl , 400 μl , 500 μl). Spread uniformly with the help of a spreader.
7. Incubate the plate invertedly overnight at 37°C and check for the transformants next morning.

The *lacZ* gene has been used in prokaryotic cloning vectors as a marker for insertion of the gene of insert. Typically, when transformations are plated on medium containing the substrate X-gal, colonies which contain vector with an insert appear white while those that contain vector without an insert are blue. In some cases, however, light blue colonies or white colonies with blue centres will appear. These colonies often contain vector with inserts.

Alpha complementation

Alpha complementation was first reported in 1967 after researchers found that coexpression of the N- and C- terminal coding sequences of the *lacZ* gene results in wild type β -galactosidase activity. In most prokaryotic cloning vectors, the small alpha fragment is present on the vector with a multiple cloning site inserted into the open reading frame of the gene. Insertion of a gene into the multiple cloning site disrupts the *lacZ* alpha open reading frame and results in an inactive, β -galactosidase protein. The substrate X-gal is hydrolyzed by functional β -galactosidase and produces a blue colour.

Screen the blues:

Many prokaryotic cloning vectors carry the a fragment of the *lacZ* gene to allow blue/white screening of colonies. Sometimes small inserts, in general 1 kb or less will not disrupt the *lacZ* a reading frame or will cause reinitiation of translation therefore allowing expression of an intact, however less active β -galactosidase protein. This results in colonies which contain inserts that are light blue or white with a blue centre. It is important, therefore, to screen these colonies for the insert, particularly if the PCR product you are cloning is small.

Selected references

- Ausubel F M, Bent R, Kingston R E, Moore D D, Seod am J G, Smith J A & Struthi K 1987 In: *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley Interscience.
- Birnboim H C & Doly J 1979 *Nucleic Acids Research* 7: 1513-1523.
- Davis L G, Dibner M D & Battey J F 1986 In: *Basic methods in molecular biology*, Elsevier, New York.
- Sambrook J & Russel 2001 In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbar Laboratory Press, New York.
- Ullman A & Perrin D 1970 In: *The Lactose Operon J.* (Bechvithe & D. Zipser eds.) Cold Spring Harbor, NY pp 143-172.

8

RAPD PCR

T E Sheeja

Introduction

RAPD stands for Random Amplification of Polymorphic DNA. RAPD is a PCR based technique that amplifies target DNA at random sites. Often in PCR a known segment of DNA is amplified with the help of designed primers. But in RAPD an unknown target sequence is amplified with primers of arbitrary sequence. Though primers anneal to several sites on the DNA, PCR shall occur only if they anneal in a particular orientation pointing towards each other within an amplifiable distance (within 2 kb) from one another.

With some randomly chosen decanucleotides no sequences are amplified. With others, the same length products are generated from DNAs of different individuals, while in some cases the banding pattern differs in case of each individual in a population. The variable bands are commonly called **random amplified polymorphic DNA (RAPD) bands**. The major reasons for this polymorphism are single base substitutions. Other sources of polymorphisms may include deletions of a priming site, insertions that render priming sites too distant to support amplification, or insertions that change the size of a DNA segment without preventing its amplification. As with any other genetic marker, some polymorphisms are clear and easy to score, while others appear ambiguous and are not useful as genetic markers. While the RAPD method is empirical, its simplicity of use and the eventual identification of some stretch of DNA, albeit unknown, to facilitate discrimination, make it a popular method of DNA typing. Several companies have now marketed RAPD kits making it a convenient fingerprinting method.

Standard protocol

RAPD protocols are relatively simple. The various components and method is detailed hereunder:

Solutions

10X buffer: 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 20 mM MgCl₂, 0.01% gelatin (the buffer is supplied ready made)

dNTP stock: 2mM each of dNTPs

Primer- 50 pmol/microlitre

Template DNA 5 nanogram/ microlitre

Polymerase 5U/microlitre

Method

Prepare a reaction mix for each primer (master mix) sufficient for all samples plus one negative control to which water is added instead of DNA. For the setup of the master mix, calculate 5 μ l of buffer, 2.5 μ l of dNTP stock, 0.2 μ l of polymerase and 37.2 μ l of H₂O for a volume of 45 μ l per sample. The final concentrations are 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, 0.001% gelatin, 100 μ M dNTPs and 0.2 μ M primer. Use a specially designated PCR pipette set. Master mixes for only a few samples are preferably made by using primers diluted to 5 instead of 50 pmol/ μ l, as this is easier to pipette. Add 5 μ l of the template DNA solution or 5 μ l of water (negative control) into labeled reaction vials and add the master mix. Mixing the contents is not essential since the high temperature will mix the samples on its own. Overlay the reaction solution with two or three drops of mineral oil (optional).

Put vials into thermocycler, and start the program: 4 min at 94°C, 45 cycles consisting of 15 s 94 °C, 45 s 36 °C, 90 s 72 °C and 4 min of 72 °C (final extension).

After amplification, the vials can be stored at 4 °C for a couple of days or at -20 °C for a longer period if necessary. The samples are then electrophoresed on a 1.4% agarose gel with TBE or TAE as a buffer and detected by staining with ethidium bromide. A suitable molecular weight marker is incorporated in one of the wells. A reaction volume of 50 μ l is used in the RAPD method described above. If the DNA yield is sufficient and if the detection method is sensitive enough smaller reaction volumes (ie. 25 μ l) are advised to save on the expensive polymerase.

Optimising RAPD patterns is laborious since many reaction components as well as any part of the PCR program can be changed with quite unpredictable effects. The brand of polymerase and thermal cycler as well as annealing temperature and primer were found to have major impact on banding pattern quality.

Primers

Primers can be purchased from several manufacturers like OPERON Technologies, Inc., UBC or Pharmacia LKB or obtained by using a DNA synthesizer. Primer concentrations are generally optimal between 0.1 and 2.0 micro molc. The majority of RAPD primers give a fragment pattern of 6-14 fragments while a few primers do not amplify.

Polymerase

A wide range of brands and types of polymerase is available for PCR. Taq Polymerase (Promega, Genei), AmpliTaq (Perkin Elmer) are the most frequently used. Super Taq (HT Biotechnology), Sure Taq, Doctor Taq (Biogene) are cheap sources. These enzymes have 5' to 3' exonuclease activity but lack 3' to 5' exonuclease or proof reading activity. Proof reading allows the enzyme to check of correct base pair matching and if necessary, to replace a false with the correct nuclotide. The initial choice of polymerase is important, switching to another type of enzyme is likely to render comparisons with previous experiments impossible. The number of polymorphic markers can be increased not only by using additional primers, but also by using another brand of polymerase.

Thermocycler

A wide variety of thermocyclers are commercially available. Most of these have 48-96 wells in a block. The machines can be programmed accordingly. Running the same programme on different thermocyclers may result in different amplification patterns. This is due to different temperature profiles in the reaction tubes. It is advisable to use the same thermocycler to maintain consistency in results.

Temperature profiles

Programmes may be optimized for each lab. Shorter periods at 94°C will prolong the life of Taq polymerase. Transition times between steps should not be too short especially important for the ramp time between the annealing and elongation temperature. The melting temperature and annealing temperature of the primer can be calculated by the general rule that $2 \times \text{no. of AT pairs} + 4 \times \text{no. of GC pairs} - 5 = \text{Annealing temperature}$. For arbitrary primers an annealing temperature of 36°C may be chosen as preliminary. In certain crops higher annealing temperature were found to yield distinct and fewer bands. Similarly significantly higher yields of DNA are obtained after 45 cycles in some cases.

Template concentration

Optimization of template concentration is extremely important for obtaining good RAPD patterns. Initially a template DNA range from 5-

500 ng may be tried, with checking for changes in fragment patterns and background levels. Negative controls without template should also be included. A reaction volume of 10-25 ng/25 microlitre is considered optimal. The concentration may be fixed by visual checking on agarose gels with a known standard. DNA can also be quantified by UV spectroscopy. A280/A260 values are measured and calculate DNA concentration using the relationship for soluble standard DNA, 1 OD at 260 nm = 50 microgram/ml.

Mg+2 concentrations

Many studies have demonstrated marked influence of Mg+2 concentrations on the RAPD patterns. While strong and reproducible bands are obtained over a wide range of Mg+2, a change in concentration often results in a qualitative change of fragment patterns. 2 mM Mg seems to be a good starting point. The chelating effect of EDTA may affect Mg concentrations if the DNA is dissolved in TE. So if not for long term storage, dissolving DNA in water is advisable.

Modifications

Several modification of the RAPD technique have been described. Modifications like using primer pairs, restriction digestion of DNA prior to or after PCR all can help to enhance the available polymorphism.

Gel electrophoresis

After the PCR the tubes are taken out and 2 microlitre 10X loading dye is added and tubes are stored at 4 °C. The amplified products are resolved on PAGE or agarose gels (1-2%) and stained with ethidium bromide or silver staining. The amplification products are usually smaller and falls within 4000 bp. The bands within the range of 100-4000 bp are considered for scoring.

General protocol

Normally agarose gels are used for checking the quality of DNA isolates and for scoring RAPD products. The procedure is given below:

1. Tape both ends of a gel box/form. Make sure that it is properly attached.
2. Find suitable combs, and adjust them so that there are a few mm between the comb and the form.
3. Weigh the appropriate amount of agarose and transfer it to suitable flask. Add the required quantity of buffer (for resolving RAPD products 1.2-2% gels are used).
4. Heat in the microwave oven until it boils vigorously (~2 min for small gels and ~5 for large). Use plastic foil (with a small hole) or a glass as a lid (NOT aluminium foil).

5. Cool under running water until it reaches $\sim 60^{\circ}\text{C}$
6. Add EtBr (**hazard!**). Treat the pipette tip as risk waste.
7. Pour the gel gently into the form. Make sure no bubbles are visible in the gel (you can puncture them using a pipette tip).
8. The gel is ready to use when it obtains a slight opaque colour. For better resolution gels may be kept in fridge for 20 minutes before starting the run. Gels may be stored overnight in fridge after wrapping them in plastic foil.

Loading gels

Before loading the gel, tape on either side of the form is removed carefully without damaging the wells. The gel is placed in the buffer so that the buffer covers the gel by some mm. Normally 1X TAE or 1X TBE buffers are used. The electrophoresis is conducted at 50-100V until the bromophenol dye front crosses 2/3 of the gel.

Scoring and analysis

1. Designate a name or a number for each RAPD marker based on the molecular size and primer used.
2. Score RAPD bands using a binary system of 0 (absence of the band) and 1 (if the band is present).
3. Data is now ready for analysis.

Specific comments

RAPD has only two possible genotypes one is absence and the other presence. They are dominant and the heterozygote and homozygote produce same banding pattern. Because the difference between the states is the presence or absence of a band, we cannot say whether the individual with the band is a heterozygote or homozygote. An individual without band is a homozygote for the null allele. RAPDs have often been criticized for low reproducibility. For optimum and consistent results, reactions to be compared should be done simultaneously and reaction conditions mentioned above should be kept a constant.

Advantages and disadvantages

RAPD is more polymorphic than RFLPs. It is simple and quick, there is no need to clone anything to mark a locus (only primer sequences are needed to define a marker, cloned probes are not necessary). Primers are universal and applicable to any species. It is the cheapest and easiest method for labs just beginning to use molecular markers. RAPD bands can often be cloned and sequenced to make SCAR (sequence-characterized amplified region) markers that are highly reproducible. Often several polymorphisms are observed per primer, and the protocols are relatively simple. In some cases, RAPD bands of differing lengths can be assigned to the same locus. In that case, these RAPD bands are codominant. RAPDs can be used as

DNA molecular markers on the genetic chromosome and may be used to detect VNTRs.

Selective neutrality and lack of reproducibility among labs are some disadvantages of RAPD. RAPDs are dominant and thus provide only half the information of codominant markers in genetic crosses. Null alleles are not directly detected by RAPDs.

Applications

RAPD can be used to identify marker linked traits without mapping of entire genome mainly through BSA (bulked segregant analysis) and analysis of NILs (near isogenic lines). RAPDs can be used to generate genus specific, species specific and population specific markers useful for identification of genus, interspecific gene flow and detection of hybrids. RAPD polymorphism among individuals of a species helps in detecting paternity and kinship relationships among large number of progenies. A large number of markers can be generated for construction of highly saturated genetic maps of complex genomes for monitoring these loci during introgression and selection programmes. The ability of the RAPD technique to reveal intra-specific variation can be used in screening for the degree of inbreeding in commercial plant and animal species to prevent an increase in the frequency of deleterious recessive alleles in populations. Breeders should be able to identify RAPD bands closely linked to the marker they wish to transfer. Scoring individuals (or groups of individuals) for the linked RAPD marker should speed the breeding process. RAPD markers linked to genes of interest can serve as starting points for chromosome walks to isolate those genes. RAPD has been used to detect DNA sequences, to diagnose genetic diseases, to carry out DNA fingerprinting to detect bacteria or viruses (particularly the AIDS virus), and to research human evolution. It has even been used to clone the DNA of an Egyptian mummy. The reproducibility of RAPD markers may be enhanced by transforming to SCAR markers and can be more advantageous in commercial breeding programs if a quick plus/minus assay can be developed for detecting presence /absence of the product. The creation of SCAR markers involves determining the DNA sequence of target RAPD fragments. Canonical SCAR markers are the products of two long PCR primers to a unique defined genomic locus, and thus identify the same genetic locus in different mapping populations. However, the effectiveness of SCAR marker methods are in part dependent on reducing the cost of obtaining the DNA sequence of the original RAPD markers.

Selected references

Kurt Weising, Hilde Nybom, Kirsten Wolff & Wieland Meyer 1995 In: *DNA Fingerprinting in Plants and Fungi*. CRC Press, Inc, Corporate Blvd., N.W., Boca Raton, Florida 33431.

9

Amplified ribosomal DNA restriction analysis- ARDRA

A Kumar, V Vinod & R Aravind

Introduction

The 16S and 23S rRNA genes have been utilized for phylogenetic analysis of both prokaryotic and eukaryotic organisms. In addition to direct comparison of the nucleic acid sequences (Woese 1987), numerous groups have used the rapid method of polymerase chain reaction (PCR) amplification of this gene (Muyzer *et al.* 1993) as well as the complete rRNA locus (Jensen *et al.* 1993) for a simple method for identification of bacterial genera and species. In these latter procedures, the amplified ribosomal gene (rDNA) is subjected to restriction endonuclease digestion; this has been termed ARDRA (Amplified Ribosomal DNA Restriction Analysis) (Vancechoutte *et al.* 1992). The resulting restriction fragment pattern is then used as a fingerprint for the identification of bacterial genomes. This method is based on the principle that the restriction sites on the RNA operon are conserved according to phylogenetic patterns.

Although ARDRA has been used for the characterization of bacterial isolates, in theory this method can also be used for analyzing mixed bacterial populations. This method involves the use of a pair of universal priming sequences for the PCR amplification of either the 16S rRNA genetic loci or the intergenic regions of the 16S and 23S rRNA genes. The latter regions exhibit a large degree of sequence, length and frequency variation because the spacer region is not well conserved. In general, ARDRA using the 16S rRNA genes will result in a simpler pattern (3-5 bands per genome when 4 base site-specific restriction endonucleases are used) than ARDRA using the 16-23S regions. Especially in the simpler case, one would expect it to be more difficult to resolve different but related organisms since they would share common restriction fragments. ARDRA using the 16-23S-rDNA region will provide a more diverse template for restriction analysis resulting in more complex band patterns and potentially higher resolution of community members. This version of ARDRA may be useful in simpler communities that are composed of closely related populations. In highly

diverse microbial communities, both primer approaches may either yield too complex a pattern or too little resolution of distinct bands. If this is the case, an alternative may be to use group specific primers for a given bacterial group or to carry out the DNA amplification under more discriminatory conditions (e.g. increasing stringency conditions during the annealing step of PCR). Further, resolution can also be obtained by hybridization with DNA probes specific for the target groups. Hence several modifications of this method can be adjusted to optimize for the community under study.

This PCR based technique provides a measure of structure and composition of microbial communities. As little as picogram quantities of DNA (1-10 cells) is sufficient for this method. In turn, community dynamics can be monitored at the genotypic level without drastically disturbing the system by sampling. Additionally this method is free of the bias of culture dependent methods, although bias can still occur if DNA is not extractable or the primers do not attach. The method is not exhaustive since it normally detects only the more dominant members. This method can also be used on pure cultures to compare their patterns with patterns derived from a community. Furthermore, band patterns of pure cultures can be used for a quick presumptive identification if they match band patterns of taxonomically described strains. Whether used with isolates or communities, matching ARDRA band patterns cannot be used to unequivocally establish identity between strains since different but closely related strains (and phenotypes) can have the same band patterns. The value of the method lies with its speed and ability to evaluate differences in dominant phylogenetic groups present in a community.

Experimental approach

A pair of highly conserved flanking sequences is used for primer binding sites to amplify the 16S ribosomal genes from microbial systems. The intragenic PCR product (~1500 bp) is then used as substrate for restriction endonuclease digestion followed by gel electrophoresis. In order to facilitate analysis and resolution (i.e. the ability to distinguish among different populations) separate digestions with three different restriction enzyme digestions is recommended. The sensitivity should be maximized by concentrating the amplified rDNA or digested rDNA product before electrophoresis. For the 16-23S rDNA intergenic region, amplification is carried out by using a pair of opposite highly conserved 16S and 23S ribosomal primers (Fig.1) followed by restriction enzyme digestion and gel electrophoresis (Arturo *et al.* 1995).

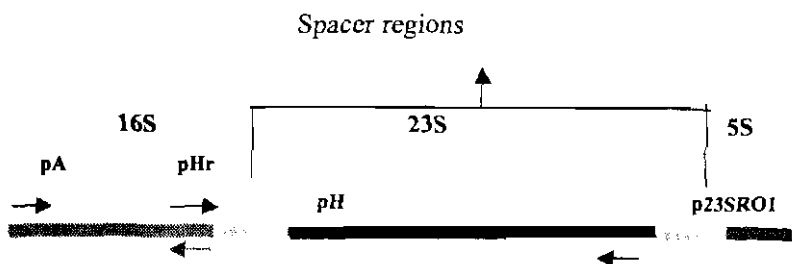


Fig.1. Ribosomal RNA operon showing approximate localization of primer binding site for PCR amplification.

Primer sequences for 16S r DNA amplification (E.coli 16S r DNA)

Primer designation	Position	Sequence (5'-3')
pA	19-38	AGAGTTTGATCCTGGCTCAG
pH	1541-1581	AAGGAGGTGATCCAGCCGCA

Primer sequences for 16-23S r DNA intergenic amplification (E.coli 16-23S r DNA)

Primer designation	Position	Sequence (5'-3')
pHr	1518-1541	TGCGGCTGGATCACCTCCTT
p23SRO1	1069-1052	GGCTGCTTCTAAGCCAAC

Template for PCR amplification

For mixed microbial cultures, it is important to maximize cell lysis before amplification since lysis efficiency is unequal for different bacterial types and physiological stages. Cell lysis by using repetitive (5 times) freeze (dry ice-ethanol bath) and thaw (80°C water bath) cycles is often satisfactory for PCR. A well optimized DNA extraction protocol is very important to ensure more representative and reproducible DNA templates for PCR.

Steps in the PCR amplification

1. Prepare master solution containing:

dd H ₂ O	86.5 µl
10X PCR Buffer	10.0 µl
100X dNTP's	1.0 µl
100X primer χ	1.0 µl

100Xprimer γ 1.0 μ l
Tag DNA polymerase (5U/ μ l) 0.5 μ l

2. Add 1 μ l (approximately 100 ng) of bacterial or community DNA, or cell suspension (approximately 10^7 to 10^8 cells). Total reaction volume is 100 μ l.
3. Amplification with the following temperature profile:

	Temperature	Time
Denature	92°C	2 min, 10 s
Melt	92°C	1 min, 10 s
Anneal	48°C	30 s (30-35 cycles)
Extend	72°C	2 min 10 s
Final extend	72°C	6 min 10 s
Soak	4°C	hold

4. After amplification, 5 to 10 μ l electrophoresed on 0.7% agarose gel made in TAE buffer to examine the amplified products.

Solutions

- 10X PCR buffer
- 500 mM KCl
- 100mM Tris-HCl, pH 8.3
- 15mM MgCl₂

Restriction endonuclease digestion and gel electrophoresis

Digestion of the amplified rDNA product with tandem tetrameric site-specific restriction endonucleases (e.g. *Haell*, *Mse*1, *Hinf* 1, *Sau*3A, *Hpa*II) should yield a restriction enzyme pattern, which can be observed by agarose gel electrophoresis. Digest with the appropriate restriction buffer at optimum temperature for 2-3 hours. The amount of DNA loaded per well is dependent of the limiting bands, which need to be resolved. The least amount of DNA that can be detected by ethidium bromide staining is about 10ng. DNA amounts greater than 100ng will not be resolved as a sharp clear band. DNA banding patterns can be performed with analytical hardware/software packages such as NTSys.

Selected references

- Arturo A, Massol-deya, David A, Odelson, Robert F Hickey & James M. Tiedje 1995 *Molecular Microbial Ecology Manual* 3.3.2: 1-8.
- Jensen M A, Webster J A & Straus N 1993 *Appl. Environ. Microbiol.* 59: 945-952.
- Muyzer G, De Waal E C & Uitterlinden A G 1993 *Appl. Environ. Microbiol.* 59: 695-700.
- Vaneechoutte M, Rossuau R De Vos P, Gillis M, Janssens D, Paepe N, De Rouck A, Fiers T, Claeys G & Kersters K 1992 *FEMS Microbiol. Lett.* 93: 227-234.
- Woese C R 1987 *Microbial Rev.* 51: 221-271.

10

DNA microarrays in microbial studies

M K Rajesh

Early detection and accurate identification of pathogens is of foremost importance in the management of diseases in many crops. Many plant pathogens are difficult to identify using morphological criteria, which can be time consuming and challenging and requires extensive knowledge in taxonomy. Molecular detection techniques can generate accurate results rapidly enough to be useful for disease management decisions.

DNA microarray has become an indispensable tool for sensitive and high-throughput analysis of transcriptome and DNA sequence variation. A DNA microarray, or gene chip, is a matrix of thousands of cDNA or oligonucleotides imprinted on a solid support. Labelled mRNA from the tissue of interest is hybridized to its sequence complement on the array to provide a measure of mRNA abundance in the sample. The hallmark of the microarray experiment is the experimental profile, the pattern of gene expression produced by the experimental sample

The power of the method lies in miniaturization, automation and parallelism permitting large-scale and genome-wide acquisition of quantitative biological information from multiple samples (Aharoni and Vorst, 2001). To perform a microarray experiment, RNA from the experimental sample(s) is first isolated and purified. The purified RNA is then reverse-transcribed in the presence of nucleotides labelled with fluorescent dyes. In the case of custom-made arrays, the fluorophores Cy3 (green) and Cy5 (red) typically are used. The two-color hybridization strategy permits simultaneous analysis of two samples on a single array. For high-density commercial arrays, non-fluorescent biotin labeled by staining with a fluorescent streptavidin conjugate typically is used. The labeled probe is fragmented and hybridized to the array, and then the array is washed and stained. Signal intensity, proportional to the amount of bound probe, is measured by scanning with a confocal laser. Background signal is subtracted from the average signal intensity for each spot on the array to generate a quantitative image. Because the sequence of each cDNA or oligonucleotide on the grid is known, the relative abundance of each transcript can be determined. Data are normalized across experiments by calculating the

variance of all genes in the sample or of a known subset of unchanging, e.g. maintenance, genes.

Principles of microarray technology

The specificity of microarray technology relies on the selective and differential hybridization of nucleic acids. Complex mixtures of labelled polynucleotides (such as cDNA derived from mRNA) are hybridized with large numbers of individual elements (e.g. unique PCR products in cDNA microarrays), attached to a solid surface. In this way information on the abundance of many polynucleotide species is gained in parallel. Labelling with fluorescent dyes possessing different excitation and emission characteristics allows the simultaneous hybridization of two samples on a single array. The strength of fluorescence emission at the two wavelengths represents the amount of a specific polynucleotide from each sample bound to the array. In this manner a single experiment provides quantitative hybridization data for hundreds to thousands of probes. For expression studies using cDNA microarrays, this approach of combining two differently labelled samples (reference and test sample) is common practice. For each gene the corresponding amount of signal in both samples can then be quantified in parallel and expression ratios obtained. This strategy, to use expression ratios instead of absolute expression levels, for the analysis of changes in gene expression, has been shown to be a very powerful one and has helped overcome a large source of experimental variation. Assuming the influence of the different dyes on the hybridization characteristics of the labelled molecules to be identical, the initial ratios between specific, differently labelled mRNA molecules should be maintained upon hybridization to the array. As a result, ratios between the two samples for each gene will then be independent of the amount of mRNA hybridized.

Microarray structure

Two variations of microarrays exist:

- (1) customized cDNA microarrays composed of cDNA or oligonucleotides, and
- (2) commercially produced high-density arrays, e.g., Affymetrix GeneChip containing synthesized oligonucleotides.

The first type of array can analyze RNA from two different samples on a single chip but requires a source of genes to be spotted onto the chip, usually expressed sequence tag clones or oligonucleotides. High-density commercial arrays provide expression analysis over a larger number of genes, but can analyze only a single sample on one chip and at considerable cost,

making them unsuitable for large scale experiments in most academic laboratories. Both types of arrays produce sensitive and accurate expression data.

Printing technologies

The printing-type technologies are based on the deposition of minute (sub-nanolitre) quantities of a DNA solution onto a solid surface (carrier). These fall into two distinct categories: contact printing (various methods for mechanical deposition) and non-contact printing (liquid delivery). Photolithographic techniques, on the other hand, can be used to synthesize oligonucleotides directly on the carrier.

Creating a cDNA microarray

Customized cDNA microarrays are fabricated by first selecting the genes to be printed on the array from public databases/repositories or institutional sources. High throughput DNA preparation, usually done by robotics systems, consists of tens of thousands of PCR reactions. Purified PCR products representing specific genes are spotted onto a matrix. Spotting is carried out by a robot, which deposits a nanoliter of PCR product onto the matrix in serial order. Nylon filter arrays largely have been replaced by glass-based arrays, typically microscope slides, which have the advantage of two-color fluorescence labeling with low inherent background fluorescence. DNA adherence to the slide is enhanced by treatment with polylysine or other cross-linking chemical coating. Spotted DNA is cross linked to the matrix by ultraviolet irradiation and denatured by exposure to either heat or alkali. The Affymetrix GeneChip is produced by a novel photolithographic method in which thousands of different oligonucleotide probes are synthesized *in situ* on the array.

Target gene selection

Selecting proper target genes is very important in nucleic acid based diagnosis. Various targets have been used for identifying pathogens, and they include:

- (i) parts of DNA encoding a particular toxin or virulence factor,
- (ii) special sequences that are revealed by subtraction hybridization among closely related species,
- (iii) small, mostly species-specific, non-coding DNA regions such as insertion elements, and
- (iv) relatively well conserved nucleic acid sequences that also serve as phylogenetic markers.

For the fourth target, there are 16S rDNA, 23S rDNA, 16S-23S rDNA

internal transcribed spacer region (ITS), and other genes that code for β -galactosidase, RNA polymerase, elongation factors Tu, F₁F₀ ATPase, RccA protein, and Hsp60 heat shock protein, *rpoB* gene. After the importance of rDNA in bacterial taxonomy for identifying evolutionary relationships among species was recognized, rDNA has been widely used as a microorganism specific genetic marker. There are many advantages for targeting probes to the 16S/18S rRNA of the small subunit of the ribosome (SSU rRNA) or to the 23S/28S rRNA of the large subunit of the ribosome (LSU rRNA). These advantages include the large amount of rRNA in most cells, *the apparent lack of lateral gene transfer, and a good length of about 1500 and 3000 nucleotides for 16S (or 18S) and 23S (or 28S), respectively, with a range of very conserved and quite variable sites.* Another important advantage is the availability of huge rRNA databases. However, 16S rDNA may be difficult to discriminate a large number of microbial strains because its sequence is conserved, which offers very little sequence diversity. Alternatively, 23S rRNA genes and the ITS region have attracted attention as target sequences. Because 23S rDNA and ITS have regions with highly specific sequences, unlike 16S rDNA, pathogens can be easily discriminated from one another. Until recently, pathogen detection that utilizes a specific target gene sequence has been limited due to sequence availability, but this limitation has been overcome because more complete sequences of the many microbial genomes are becoming available.

Sample preparation and hybridization

In order to prepare a sample that can be hybridized with target probes, which were immobilized on the solid substrate, the DNA is boiled or asymmetric PCR is carried out. In general, DNA is chemically modified by incorporating fluorescent nucleotides or end-labeled by using a one-side primer with 5'-fluorescein. The amplified products are hybridized on a slide in hybridization solution. After washing, the slides are air-dried, and by using the confocal laser scanner, they are scanned under a wavelength that is compatible in fluorescent excitation.

Data Management

Once the hybridized chip is scanned, data flow occurs through the following steps. Data are collected and saved as both an image and a text file. Of critical importance is that precise databases and tracking files be

maintained regarding the spot configuration of all chips. These contain information on the location and names of genes arrayed on each chip. The saved files are imported to software programs that perform image analysis and statistical analysis functions. Finally, the data are mined for induced or repressed genes, patterns of gene expression, and temporal relationships of expression under different experimental conditions. A significant challenge exists in making sense of the vast quantity of data generated by microarray experiments. There is no single tool that meets all of the needs of the microarray researcher. Collections of software programs are used to perform a multitude of tasks, including data tracking, image analysis, database storage, data queries, statistical analysis, multidimensional visualization, and interaction with public databases on the Internet. Basic spreadsheet programs can be adapted to answer questions regarding magnitude of change in gene expression. However, limitations often arise as a result of inadequate memory capacity for managing the enormous data sets. More sophisticated analytical tools, including cluster analysis, self-organizing maps, and principle component analysis, have been applied to biological data to extract higher-order relationships embedded in expression patterns.

Image analysis

The sample hybridized to each element is visualized by fluorescence detection. Both confocal scanning devices and CCD cameras are being used for this purpose. Fluorescence emission from the microarray is converted into a digital output for each dye, and is stored as a separate image file. Image analysis software (P-scan, ScanAlyze, Spotfinder) is then used for quantification of individual array elements. A grid is superimposed over the image and the average (or median) pixel intensities for each element is calculated for both dyes. Background fluorescence is then subtracted from the raw data. Although fluorescent signal measured (directly) on areas between the array elements are often employed for background, it is more approximate to use signals from foreign array elements that have been included in the array for this purpose. Subsequently the figures are normalized to correct for channel specific effects such as differences in quantum yield of the dyes and unequal labelling efficiencies of the samples. Normalization also corrects for any unwanted differences in the amount of sample used.

Several ways of normalization are being used:

- (i) overall hybridization signal
- (ii) use of housekeeping genes

- (iii) spiking with a foreign mRNA species that has been included on the array for this specific purpose.

None of these methods alone is sufficient to ensure a satisfactory normalization and often a combination of these methods is employed.

Data exploration

Several standard statistical techniques are currently being used to interpret microarray data, including hierarchical clustering, principal component analysis (PCA) and self-organizing maps (SOM). These are all focused on grouping genes (or samples) together which show similar behaviour. This type of analysis, with large data sets, can provide novel perspectives on cellular regulatory mechanisms and can associate expression of unknown genes with a putative function.

Hierarchical clustering of gene expression data in combination with false colour coding of the expression levels has become a popular way of data analysis and presentation. With this technique genes are grouped in clusters based on similarity between their expression profiles. In a bottom-up approach genes are joined to form nodes, which in turn are then further joined. Joining proceeds until all genes are combined in a single hierarchical tree.

Application of microarrays in microbial studies

DNA microarrays used in microbial ecology studies are still not common as compared to other research area.

Oligonucleotide microarrays were used to detect 16 s rRNA in soil extracts without prior amplification of the targets by PCR in analytical studies of microbial diversity in different environments (Small *et al.* 2000) or for all recognized lineages of sulphate-reducing prokaryotes in both natural and clinical environments (Loy *et al.* 2002). DNA microarray has also been applied for the detection of functional genes involved in the nitrogen cycle in the environment (Wu *et al.* 2001; Taroncher-Oldenburg *et al.* 2003). Direct profiling of environmental microbial populations by thermal dissociation analysis of native rRNAs hybridized to oligonucleotide microarrays was done by El Fantroussi *et al.* (2003). Difference in the apparent distribution of nitrite reductase (*nirS* and *nirK* genes), ammonia mono-oxygenase (*moaA*) genes and methane mono-oxygenase (*pmoaA*) genes could be made between marine sediment samples and soil samples. DNA microarray-based genome comparison of a pathogenic and a non-pathogenic strain of *Xylella fastidiosa* could delineate genes important for bacterial virulence (Koide *et al.* 2004).

A DNA microarray suitable for simultaneous detection and discrimination between fish pathogens based on 16S rDNA polymorphisms was developed by Warsen *et al.* (2004). Maynard *et al.* (2005) demonstrated the feasibility of using DNA microarrays in the detection of waterborne pathogens. A disposable microarray was developed for detection of upto 90 antibiotic resistance genes in gram-positive bacteria by hybridization (Perreten *et al.* 2005). An oligonucleotide microarray detecting 189 *Escherichia coli* virulence genes or markers and 30 antimicrobial resistance genes was designed and validated using DNA from known reference strains. This microarray was confirmed to be a powerful diagnostic tool for monitoring emerging *E. coli* pathotypes and antimicrobial resistance, as well as for environmental, epidemiological, and phylogenetic studies including the evaluation of genome plasticity (Bruant *et al.* 2006).

Limitations of the microarray technology

Possible constraints of the DNA microarray in the field of environmental research include:

- (i) The expense of microarray printing and imaging equipment,
- (ii) The time and labour required for manual handling, nucleic acid purification and associated volume reduction,
- (iii) The diverse and complex nature of environmental samples
- (iv) The inefficient purification or concentration of nucleic acids at low concentrations, especially in environmental samples,
- (v) The co-extraction of inhibitory compounds that interfere with subsequent molecular manipulations, and
- (vi) The problem of secondary structures within single-stranded RNA or DNA.

Future perspectives

Microarray technology provides a highly efficient and rapid method of analysis in microbial ecology studies. As the microarray technology is still in early stages of development in the field of microbial studies, intensive studies with careful selection and screening of probes and vigorous and systematic optimization of hybridization conditions should continue to modify and improve the microarray technology. Technical improvements in microarray technology continue to take place. Current areas of development include improving RNA amplification methods to allow

analysis of smaller amounts of RNA and, eventually, single-cell expression analysis. Through these efforts, the full application of this powerful molecular technology in microbial studies is promising.

Selected references

- Aharoi A & Vorst O 2001 *Plant Mol. Biol.* 48: 99-118.
- Bruant G, Maynard C, Bekal S, Gaucher I, Masson L, Brousseau R & Harel J 2006 *Appl. Environ. Microbiol.* 72: 3780-3784.
- El Fantroussi S, Urakawa H, Bernhard A E, Kelly J J, Noble P A, Smidt H Yershov G M & Stahl D A 2003 *Appl. Environ. Microbiol.* 69: 2377-2382.
- Koide T, Zaini P A, Moreira L M, Vencio R Z, Matsukuma A Y, Durham A M, Teixeira D C, El-Dorry H, Monteiro P B, da Silva A C, Verjovski-Almeida S, da Silva A M & Gomes S L 2004 *J. Bacteriol.* 186: 5442-9.
- Loy A, Lehner A, Lee N, Adamczyk J, Meier H, Ernst J, Schleifer K H & Wagner M 2002 *Appl. Environ. Microbiol.* 68: 5064-5081.
- Maynard C, Berthiaume F, Lemarchand K, Harel J, Payment P, Bayardelle P, Masson L & Brousseau R 2005 *Appl. Environ. Microbiol.* 71: 8548-8557.
- Perreten V, Vorlet-Fawer L, Slickers P, Ehrlich R, Kuhnert P & Frey J 2005 *J. Clin. Microbiol.* 43: 2291-2302.
- Small J, Call D R, Brockman T M, Straub T M & Chandler D P 2001 *Appl. Environ. Microbiol.* 67: 4708-4716.
- Taroncher-Oldenburg G, Griner E M, Francis C A & Ward B B 2003 *Appl. Environ. Microbiol.* 69: 1159-1171.
- Warsen A E, Krug M J, LaFrentz S, Stanek D R, Loge F J & Call D R 2004 *Appl. Environ. Microbiol.* 70: 4216-4221.
- Wu L Y, Thompson D K, Li G, Hurt R A, Tiedje J M & Zhou J 2001 *Appl. Environ. Microbiol.* 67: 5780-5790.

11

Isoenzyme analysis

K S Krishnamurthy

In isozyme analysis, the sample extract is electrophoresed in starch or poly / acrylamide buffered (Non –denaturing) slab at a low temperature (4-8°C). Each lane should be loaded with small equal amount of proteins, (50-100µg in 30-50µL of the extract). After electrophoresis the gel is removed from the glass plate and is incubated in a solution containing all the reactants for the enzyme to act. The isozymes are visualized as achromatic or chromatic bands on the gel after the reaction is completed

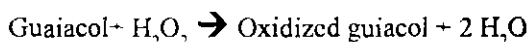
Peroxidase

Peroxidase (POD) catalyses the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydroquinones etc.

Principle

Guaiacol is used as substrate for the assay of peroxidase

POD



The resulting oxidized (dehydrogenated) guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product is a measure of the POD activity and can be assayed spectrophotometrically at 430nm.

Peroxidase isozyme

Peroxidase can be visualised using benzidine. Prepare the staining solution for peroxidase by mixing the following reagents.

Benzidine	:	1.4g
Acetic acid	:	9ml
H ₂ O ₂ (1 %)	:	50ml
water	:	40ml

Place the gel in the above solution, keep in dark till the bands appear (30 minutes to two hours). Stop the reaction by immersing the gel in 0.67% NaOH/ 7% acetic acid for ten minutes. Peroxidase isozymes appear as dark bands.

Alternatively, soak the gel for 15-30 minutes in 15 mM Sodium phosphate buffer (pH 6.0) containing 1 mM H_2O_2 and 0.1 mM guaiacol. Peroxidase isozymes appear as dark bands. Rinse the gels in deionised water and fixed in 7% CH_3COOH .

Super oxide dismutase (SOD)

SOD is the first line of defense against injury caused by Active oxygen species, catalyzing the dismutation of oxygen to H_2O_2 and molecular oxygen.

According to the metal co-factor and by the enzyme, three major SOD types have been described, Fe-SOD, Mn-SOD and Cu-Zn-SOD. In plants, Cu-SOD is the most abundant and has been localized in the cytosol, chloroplast, peroxisome and apoplast. Imposition of biotic/abiotic stress may give rise to an excessive concentration of AOS (Active oxygen sp.) resulting in oxidative damage at a cellular level. To mitigate and repair damage initiated by AOS, plants have developed a complex antioxidant system.

SOD Isozymes

Stain the gel by mixing the following.

Tris	:	0.05M
Na-EDTA	:	7.5 mg
Riboflavin	:	4 mg
NBT	:	10mg (add water and make volume up to 100ml)

Cover the gel with the above stain solution and incubate at 37 °C for 20 minutes in dark. Remove from dark and provide fluorescent light for 10-15 minutes. SOD isozymes appear as achromatic bands in a blue background on the gel.

Enzyme extraction

All extractions should be done under ice cold conditions using a pre-chilled pestle and mortar. Clean the plant material thoroughly by rubbing with cotton to remove the particles adhering to the leaf surface. Grind 0.5

g of the leaf material in 5 ml of 0.1 M phosphate buffer (pH 7.2) containing 5 % PVP, 0.2 M ascorbic acid and 0.1 % sodium metabisulphite. Filter the homogenate through 4 layers of cheesecloth and centrifuge at 10000 rpm for 20 minutes. Collect the supernatant and store at -20°C. Estimate the protein concentration in the supernatant and use 50-100 µg protein for loading.

Selected references

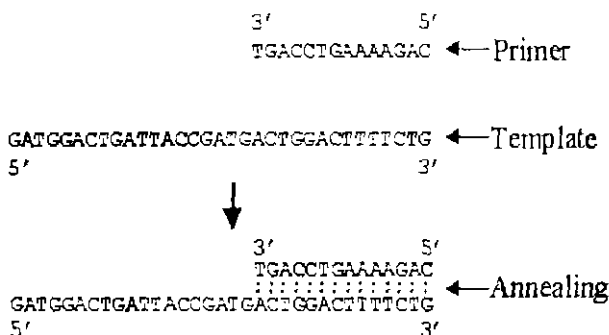
- Bucno P, Vasele J, Gimenez G & Id Rio L A 1995 *Plant Physiology* 98: 331-336.
- Foyer C H & Mullineaux P M 1994 Cause of photooxidative stress and amelioration of defense systems of plants. Boca Raton, C. L, CRC Press.
- Shimon M & Renveni R A 1988 *Annal Biochem.* 175: 35.

12

Primer designing

S Balaji & Santhosh J Eapen

A primer is a short synthetic oligonucleotide which is used in many molecular techniques from PCR to DNA sequencing. These primers are designed to have a sequence which is the reverse complement of a region of template or target DNA to which we wish the primer to anneal.



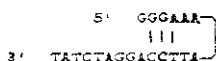
Analysis of primer sequences

When designing primers for PCR, sequencing or mutagenesis it is often necessary to make predictions about these primers, for example melting temperature (T_m) and propensity to form dimers with itself or other primers in the reaction. Some online programs will perform these calculations on any primer sequence or pair.

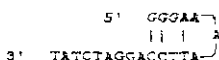
The programs will calculate both the T_m of the primers as well as any undesirable pairings of primers. When primers form hairpin loops or dimers, less primer is available for the desired reaction.

Hairpin

Oligo, 3 bp (Loop=4), delta G = -0.1 kcal/m



Oligo, 2 bp (Loop=3), delta G = 2.1 kcal/m



Self-Dimer

```

4 bp, delta G = -6.6 kcal/m (bad!) (worst= -36.6)
5' GGGAAAATTCAGGATCTAT 3'
      (|||| |||)
3' TATCTAGGACCTTAAAAGGG 5'

```

```

4 bp, delta G = -5.4 kcal/m (bad!) (worst= -35.6)
5' GGGAAAATTCAGGATCTAT 3'
      (||||)
3' TATCTAGGACCTTAAAAGGG 5'

```

The choice of the length of the primers and their melting temperature (T_m) depends on a number of considerations. The melting temperature of a primer (not to be confused with the melting temperature of the template DNA) is defined as the temperature at which half of the primer binding sites are occupied. Primers that are too short would anneal at several positions on a long DNA template, which would result in non-specific copies. On the other hand, the length of a primer is limited by the maximum temperature allowed to be applied in order to melt it, as melting temperature increases with the length of the primer. Melting temperatures that are too high, i.e. above 80°C, can cause problems since the DNA polymerase is less active at such temperatures. The optimum length of a primer is generally from 15 to 40 nucleotides with a melting temperature between 55°C and 65°C.

Also keep in mind that most oligonucleotide synthesis reactions are only 98% efficient. This means that each time a base is added, only 98% of the oligos will receive the base. This is not often critical with shorter oligos, but as length increases, so does the probability that a primer will be missing a base. This is very important in mutagenesis or cloning reactions. Purification by HPLC or PAGE is recommended in some cases.

Basic rules

1. Primers should be 17-28 bases in length.
2. Base composition should be 50-60% (G+C).
3. Primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming.
4. T_m s between 55-80°C are preferred.
5. 3' ends of primers should not be complementary, as otherwise primer dimers will be synthesized preferentially to any other product.

6. Primer self-complementarity (ability to form 2° structures such as hairpins) should be avoided.
7. Runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided.

Designing degenerate oligonucleotides

Sometimes *degenerate primers* are used. These are actually mixtures of similar, but not identical, primers. They may be convenient if the same gene is to be amplified from different organisms, as the genes themselves are probably similar but not identical. The other use for degenerate primers is when primer design is based on protein sequence. As several different codons can code for one amino acid, it is often difficult to deduce which codon is used in a particular case. Therefore primer sequence corresponding to the amino acid isoleucine might be "ATH", where A stands for adenine, T for thymine, and H for adenine, thymine, or cytosine. Use of degenerate primers can greatly reduce the specificity of the PCR amplification. This problem can be partly solved by using touchdown PCR.

A group of degenerate oligonucleotides contain related sequences with differences at specific locations. These are used simultaneously in the hope that one of the sequences of the oligonucleotides will be perfectly complementary to a target DNA sequence. One common use of degenerate oligonucleotides is when the amino acid sequence of a protein is known. One can reverse translate this sequence to determine all of the possible nucleotide sequences that could encode that amino acid sequence. A set of degenerate oligonucleotides would then be produced matching those DNA sequences. The following link will take you to a program that will perform a reverse translation (<http://arbl.cvmbs.colostate.edu/molkit/rtranslate/>). For example, the amino acid sequence shown in below could be encoded by the following codons.

Asp-Glu-Gly-Phe-Leu-Ser-Tyr-Cys-Trp-Leu-Pro-His-Gln

GATGAAGG**T**TTTCTTTCTT**T**ATTGTTGGCTT**C**CTCATCAA

One could then select the 14 base sequence (in bold) to generate a smaller set of degenerate oligonucleotides. Each oligonucleotide in the set would have one base changed at a time. A total of 32 unique oligonucleotides would be generated.

TATTGTTGGCTTCC

TACTGTTGGCTTCC

TATTGCTGGCTTCC

TACTGCTGGCTTCC *etc.*

When ordering degenerate oligonucleotides, you just let the company know that you want a mixture of nucleotides added at a specific position using the code below. By adding the mixture, oligos will incorporate one of the bases, leading to a mixture of oligonucleotides.

Standard	MixBase Definitions
R	A, G
Y	C, T
N	A, C
K	G, T
S	C, G
W	A, T
H	A, C, T
B	C, G, T
V	A, C, G
D	A, G, T
N	A, C, G, T

Designing primers - A checklist

1. Are annealing temperatures of each pair of primers for a corresponding PCR product similar?
2. Does the primer have an annealing segment? A tag segment? A restriction enzyme site? Extra bases to help cut the restriction site?
3. Is the primer in the correct orientation (if necessary, is it in reverse complement? If necessary, are the extra bases in reverse complement?) Is the annealing temperature at least 50°C?

Materials required

- (a) Gene of interest to amplify
- (b) Softwares
 - Sequence retrieval
<http://www.ncbi.nlm.nih.gov/>
 - Multiple sequence alignment
<http://www.ebi.ac.uk/clustalw/>

- Oligonucleotide properties calculation (T_m, GC% etc.,)
<http://www.basic.northwestern.edu/biotools/oligocalc.html>
- Secondary structure analysis
<http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html>
- Primer specificity
<http://www.ncbi.nlm.nih.gov/blast>

Procedure

Try to design primers for the consensus sequence or the variable sequences from the pattern you got from multiple sequence alignment.

The choice of consensus sequence or the variable sequences is depending on your interest to design universal or genus specific primer.

- I. Make a Multiple Sequence Alignment (MSA) of the sequences of your interest.
- II. Check approximately the number of conserved bps.
- III. Copy conserved stretch of residues of different combinations and check for the following.
 - Length
 - GC%
 - Check the ends
 - T_m
 - Self-complementarity

After verifying the length, T_m etc.

- IV. Use any dimer analysis software to check your primers. If not then you can also check the secondary structure of the mRNA, by using *mfold* software.

Selected references

- Dieffenbach C W, Lowe T M J, Dveksler G S 1995 In: *PCR Primer- A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, pp. 133-155.
- Innis M A & Gelfand D H 1994 In: *PCR protocols, A Guide to Methods and Applications*, CRC Press, London, pp. 5-11.
- Sharrocks A D 1994 In: *PCR Technology, Current Innovations*, CRC Press, London, pp. 5-11.
- Kwok S, Kellog D E, McKinney N, Spasic D, Goda L, Levenson C & Sninsky J J 1990 *Nucleic Acids Res.* 18: 999-1005.
- Linhart C & Shamir R 2002 *Bioinformatics* 18 Suppl 1: p. S 172-181.

Reference websites

<http://www.primerdesign.co.uk/>

<http://www.mcb.uct.ac.za/pcroptim.htm>

<http://dbb.nhri.org.tw/primer/index.html>

<http://bibiserv.techfak.uni-bielefeld.de/genefisher/>

http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

<http://seqcore.brcf.med.umich.edu/doc/dnaseq/primers.html>

http://bioweb.uwlax.edu/GenWeb/Molecular/Seq_Anal/Primer_Design/

[http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/
Default.aspx](http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx)

<http://www.premierbiosoft.com/primerdesign/index.html>

13

Construction of phylogenetic trees

S Balaji & Santhosh J Eapen

A phylogenetic tree is a specific type of cladogram where the branch lengths are proportional to the predicted or hypothetical evolutionary time between organisms or sequences. Cladograms are branched diagrams, similar in appearance to family trees that illustrate patterns of relatedness where the branch lengths are not necessarily proportional to the evolutionary time between related organisms or sequences. Bioinformaticians produce cladograms representing relationships between sequences, either DNA sequences or amino acid sequences. However, cladograms can rely on many types of data to show the relatedness of species. In addition to sequence homology information, comparative embryology, fossil records and comparative anatomy are all examples of the types of data used to classify species into phylogenetic taxa. So, it is important to understand that the cladograms generated by bioinformatics tools are primarily based on sequence data alone. Given that, it is also true that sequence relatedness can be very powerful as a predictor of the relatedness of species.

Methods

- Neighbor-joining
- Minimum Evolution
- Maximum Parsimony
- Maximum Likelihood
- UPGMA

Materials required

- (a) Conserved genes from organisms (Nucleotide sequence data)
- (b) Softwares
 - Nucleotide sequence retrieval
<http://www.ncbi.nlm.nih.gov/>
 - Protein sequence retrieval
<http://www.expasy.org>
 - Multiple sequence alignment
<http://www.ebi.ac.uk/clustalw/>

- Phylogenetic tree
<http://www.genebee.msu.su/clustal/basic.html>
- Advanced phylogenetic tree construction
<http://evolution.genetics.washington.edu/phylip/software.html>

Procedure

Try to construct phylogenetic tree for the consensus sequence from multiple sequence alignment.

- I. Get the complete coding sequence for gene of your interest and perform a BLAST search.
- II. Collect similar sequences from different taxa (or collect 8 species).
- III. Make a Multiple Sequence Alignment (MSA) of the sequences by using Clustal X (Or else follow IV).
- IV. Go to the ClustalW server at EBI: <http://www.ebi.ac.uk/clustalw/> and enter all your sequences together in the provided field. Perform the multiple alignment. Copy the entire window's contents to a NotePad document.
- V. Go to the online Phylip page at: <http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html>.
- VI. Create two different phylogenetic trees for the multiple alignment you've created using the programs **DNAdist** with **neighbor** (distance matrix) and **fastDNAm1** (maximum likelihood) under **Programs for molecular sequence data**.
- VII. Print out both trees in **drawgram** format. Make sure you keep track of which method created which tree.

Which of these two methods do you think is more likely to produce correct results? Why?
- VIII. On each tree, label each node with the name of its corresponding species.

Explain briefly two of the differences you can see between the phylogenies you obtained.
- IX. Check the status of your taxa or the organism/ gene of your interest among the 8 species you have inputted to make a phylogenetic tree.

Reference website

<http://evolution.genetics.washington.edu/phylip/software.html>

III

CHARACTERIZATION

Biological characterization of plant pathogens

M N Venugopal

Diseases caused by fungi, bacteria, virus and virus like agents are responsible for heavy losses world – wide. To control these diseases, it is important to identify the causal agent and to determine its characters. So long as the causal agent remains a mystery, the presence of a disease can be demonstrated only by biological tests to plant indicators. Though recent advances in diagnostic methods made the characterization easy and reliable, biological characterization methods viz. study of symptoms in field and simulated conditions, isolation and identification of associated pathogens, host range, transmission and identification of indicator plants play a important role in understanding disease and disease complexes. Biological characterization continues to be essential for identifying and characterization of new agents, new races and diseases also in formulating disease management strategies.

Disease symptoms

Symptomatology is the study of symptoms and signs as evidence in disease diagnosis. Each disease or several diseases on a common host produce a characteristic symptom, which are the visible effects of pathogen on plant. Individual diseases are recognized largely by characteristic symptoms. Familiarity with symptoms and signs is essential in disease and pathogen identification. Adverse factors like drought, cold, heat, pesticides, herbicides and lightening cause look-like symptoms. Careful study of symptoms and verification from documented sources is the basic step in studying the disease and associated pathogens. The specimens can be reexamined to check their identity with biological collections.

Following are the general disease symptoms produced by various groups of pathogens.

Root symptoms

Injury to the root system often includes yellowing, stunting, or wilting of above ground parts. Wash off the roots when possible and look for the following:

- Small discolored or dead areas (fungi)
- General death of the feeder roots or the entire root system (fungi and root lesion or burrowing nematode)
- Discoloration of the vascular tissue in the crown and lower stem (fungi, such as *Verticillium* spp. and *Fusarium* spp., bacteria)
- Galls on roots (crown gall (caused by bacterium *Agrobacterium tumefaciens*, fungal diseases such as club rot of cabbage, root knot nematodes)

Symptoms on storage organs

Storage organs include tubers, bulbs, and corms. Symptoms on these organs include:

- Discolored or dead areas that go deep into storage organs (fungi, bacteria, nematodes)
- Dry rots (fungi)
- Soft rots accompanied by strong, repulsive odors (bacterial pathogens, such as *Erwinia* spp.) (Frequently, bacterial soft rots move into tissue originally attacked by pathogenic fungi, making diagnosis of the original pathogen difficult.)
- Scabby lesions on potato skins (bacteria such as common scab)
- Galls on storage organs (fungi, nematodes)
- Internal problems (several viruses or bacteria, such as ring rot of potato)

Seedling diseases

- Seedlings fail to emerge, or fall over and die (damping-off caused by fungi such as *Rhizoctonia*, *Pythium*, and *Fusarium*)
- Dead areas on cotyledons or stems (fungi, bacteria)

Leaf symptoms

- Leaf discoloration or yellowing in localized or distinct patterns (viral)
- Necrotic (dead) areas on leaves, often containing fruiting bodies (fungi)
- Necrotic areas on leaves, often with water-soaked margins (bacterial)
- Small rusty-red, brown or black spots and stripes (fruiting bodies of rusts and smut fungi)

- Leaf distortion (elongated, dwarfed, thickened, etc.) (viral)
- Leaf galls (fungi such as peach leaf curl and oak leaf blister, insects)
- White, powdery substance on leaves (powdery or downy mildew)
- Wilting (vascular wilt fungi, root rot pathogens, bacteria, drought)

Stem, branch, and trunk disorders

- Cankers and complete or partial death of woody stems or branches (fungi and bacteria)
- Sticky ooze from trunks and branches (bacteria, mechanical injury, stress, boring insects, sapsuckers (birds))
- Large conks and bracket-like fruiting structures on trunks and branches (wood-rotting fungi)
- Galls or swellings on lower trunk and/or branches (crown gall bacterium, white pine blister rust)
- Witches' brooms or excessive branching (fungi, mistletoes, phytoplasmas)
- Extreme distortion, enlargement, and flattening of stems or branches (physiological/genetic condition known as fasciation).

Flower symptoms

- Odd color changes (often in a mosaic pattern) and/or distortion (viruses)
- Death of flower parts (fungi such as gray mold (*Botrytis* spp., bacteria))
- Individual flowers or seeds converted into masses of black spores (corn smut)
- Flowers that are green and smaller and more dense than normal (phytoplasma)

Fruit symptoms

- Fruit decays, rots, and superficial spotting or russetting (fungi). Important diagnostic symptoms include specific color of rotted tissue, firmness of the tissue, and signs such as spores or fruiting structures
- Discolorations and malformations (viruses)
- Discrete spots on fruit or soft rots in storage (bacteria).

Pathogenicity tests or bioassays

The ultimate verification of a plant pathogen generally requires a pathogenicity tests, but these tests are to be carried in ideal field or controlled simulated field conditions. In the field samples several micro organisms may be isolated from a disease specimen. To find out the real pathogen the following Koch's postulates are to be followed.

1. Determine whether suspected pathogen is always associated with the disease (symptom complex)
2. Isolate the suspected pathogen, grow it in pure culture on an artificial medium and describe it.
3. Inoculate the organism on to a healthy plant of the same variety or species and determine whether the symptoms obtained are the same as those of the original disease.
4. Reisolate the pathogen from the inoculated plant and compare with the original isolate. This rule was added to the rules originally formulated by Robert Koch (1882) by the American Plant Pathologist E.F. Smith. This confirms that the *organism actually spread and multiplied in the host*. For obligate parasites, downy mildews, rusts, viruses and virus like agents, which cannot be, cultured on artificial media, these postulates cannot be applied. However, by following *different transmission techniques contagious nature of disease causing agents can be confirmed which is the main object of Koch's postulates.*

Isolation and characterization of pathogens

Bacterial and fungal pathogens often have to be isolated and cultured from diseased plant specimens before these can be identified. Pathogens capable of saprophytic growth can generally be grown in culture although some of these are fastidious in their requirement. Isolation of fungi from plant material is usually achieved by placing small portion of tissue on to a suitable agar medium in sterile Petri plates. Spores removed directly from fruiting bodies with a fine needle can also be placed on the surface of the agar.

Many saprophytic fungi and bacteria grow on or contaminate plant tissue as secondary colonizers of disease lesions. Proper surface sterilization and by use of selective media the target pathogen can be isolated and brought to pure culture. The pure cultures can be obtained from the primary inoculation plates by colonies initiated by single spore or hyphal tip. Baits

and selective media are other important methods used for selective isolation and quantification of pathogens from soil. Many fungi grow well in laboratory conditions and they can be induced to sporulate under near ultraviolet light (black light).

The isolated fungi can be identified by staining with cotton blue and lacto-fuchin and observing under light microscope. Many obligate pathogens like rusts and downy mildews are purified through repeated inoculations and maintained on live hosts or on the semi synthetic media/respective host calli.

Characterization of fungal pathogens

Colony characters, morphology of reproductive structures, branching pattern of mycelium, host range, virulence, biometry and cytology are the parameters used in characterizing fungal pathogens and their strains. The differential expressions of disease on a set of hosts are often used to differentiate and to identify the pathogens and their races. In obligate parasites like rusts and downy mildews, the reaction on different host genotypes / varieties is widely used to characterize species and their races. In *Sclerospora graminicola* an obligate fungal pathogen, four pathotypes are identified based on their differential reactions with bajra genotypes, morphology, biometry, cytology and serological relationship. Similarly brown rust (*Puccinia recondata* f sp *tritici*), black rust (*P. graminis* f sp *tritici*) and yellow rust (*P. striiformis* f sp *tritici*) and their races are identified on their reaction on a set of differentials.

Isolation of bacteria

Washing diseased samples in sterile water, quick dip into 10% sodium hypo chlorate is sufficient for surface sterilization. Routine surface sterilization kills associated bacteria and only for thicker plant parts surface sterilization in 70% alcohol is done prior to isolation. Streaking of suspension containing bacterial ooze obtained from infected plant part is done during initial stage of isolation. Sub culturing is done through well separated colonies. Nutrient agar is a good general-purpose medium for isolation of pathogenic bacteria.

Observation of bacteria in the infected tissue can be facilitated using 0.1% Toluidine blue o under x 400 magnification. If present it may be possible to see motile bacteria around the cut edge tissues, bacteria are stained a deep blue, plant tissues a paler greenish-blue. One of the major

divisions of bacteria is that between those can retain a complex of crystal violet and iodine against elution with ethanol (gram positive) and those that cannot (gram negative). The gram stain is useful as a primary determinative character for many plant bacteria.

Characterization

Pathogenic isolates of bacteria are characterized based on colony colour, morphology, phenotypic characters, utilization of carbon sources, nitrite metabolism and pectolytic activity. Five biovars of *Ralstonia solanacearum* infecting cup plants are recognized based on the above characters. *Xanthomonas campestris* pv *vesicatoria*, causal agent of bacterial spot of tomato and pepper for 70 years was considered homogeneous group. Presently genetically and phenotypically distinct two groups are recognized based on amylolytic activity, reaction on differential hosts (tomato races TI and TII), reaction patterns with monoclonal antibodies, DNA restriction profiles and DNA-DNA hybridization.

Maintenance of virus cultures

Virus and virus like organisms are generally maintained on the respective hosts or propagation hosts under controlled conditions.

Characterization of virus and virus like agents

Combination of several criteria such as characteristic symptoms, transmission through sap, vectors, dodder, graft, inclusion bodies, purification methods, particle morphology, host range, typical reaction on indicator hosts are used preliminarily to characterize associated virus. In mechanically transmissible viruses, the virus distribution in different host parts and virus concentration is determined through serial dilution and bioassay on indicator host. Host range and differential expression of symptoms on set of indicator hosts is another widely employed key to characterize the virus. In vector transmitted viruses, the different combination of pre-acquisition fasting, acquisition feeding, serial transmission, post acquisition fasting, different periods of post acquisition feeding are used to characterize virus into non-persistent, semi-persistent and persistent viruses.

Biological indexing on members of Solanaceae, Cucurbitaceae, Chenopodiaceae, Amaranthaceae and many woody plants remains as essential tool for identifying and characterization of new agents and diseases and also diagnosis in certification programmes. In many cases where the pathogen involvement is not known, the presence of disease can be demonstrated only by biological transmission to plant indicators. Besides virus characterization, indicator hosts can be used to identify virus and their strains. Cross protection by virus strains is also used to identify related virus strains in Tristiza infecting citrus and TMV infecting tomato.

In spite of advances in the immuno diagnostics and molecular techniques several viruses like viruses infecting potato, tomato, orange, stone fruits, chilli, cardamom etc biological indexing on set of indicator hosts is still in practice for virus diagnosis in plant and seed certification programmes.

Conclusion

Despite disadvantages of being laborious, time consuming and skill demanding, biological characterization remains the primary and compulsory approach in identification, characterization and diagnosis of plant pathogens. In the future, even with everchanging advances in molecular techniques, the biological characterization continues to play the prime role in diagnosis of pathogens in seed and plant health programmes. Cost effectiveness, access to reagents and sophisticated analytical techniques are other factors favouring the application of traditional biological characterization methods in disease management strategies.

Selected references

- Agrios G N 2005 Plant Pathology, 5th edition, Elsevier Academic Press, Gainesville, USA.
- Barnet H L & Hunter B B 1998 Illustrated Genera of Imperfect Fungi, 4th Edition, APS Press, St. Paul, Minnesota, USA.
- Brunt A, Crabtree K, Dallwitz M, Gibbs A & Watson L 1996 Viruses of Plants: Descriptions and lists from the VIDE database, CAB International Publishing, UK.
- CAB International 2004 Crop Protection Compendium, CAB International Publishing, Wallingford, UK.
- Green S K & Kim J S 1991 *Tech. Bulletin* 18, AVRDC, Taiwan.
- Jones J B & Stall R B 1998 *Ann. Review Phytopathol.* 36: 41-58.
- Mehan V K & McDonald D 1995 *Tech. Manual 1*, ICRISAT, Patancheru, India.
- Nagarajan S, Nair S K, Bahadur P & Kumar J 1986 In: *Wheat Pathology and Improvement*, IARI, Regional Station, Flowedale, Shimla.
- Noordam D 1973 Identification of Plant Viruses, Methods and Experiments. Centre for Agriculture Publishing and Documentation, Wageningen.
- Paul Khurana S M 1992 *Tech. Bullt.* 35, CPRI, Shimla, India.
- Schad N W, Jones J B & Chan W 2001 Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd edition, APS Press, St. Paul, Minnesota, USA.
- Shetty S A, Shetty H S & Mathur S B 1995 *Tech. Bullt.* Dept. of Studies in Applied Botany, Mysore.
13. The Commonwealth Mycological Institute 1968 Plant Pathologists Pocket Book, 3rd edition, Kew, Surrey, England.

15

Characterization of pathogens based on isozymes and allozymes markers

Shamina Azeez & B Chempakam

Accurate identification and early detection of pathogens is the corner stone of disease management in many crops. One aspect of this is to study the pathogen diversity in the environment. Pathogens have traditionally been differentiated at species and subspecies level using biochemical and immunological characters. However, a number of typing methods, based on molecular analyses have recently been used to study the diversity with varying degrees of success.

Intra- and inter specific variability in pathogens has been traditionally studied using morphological parameters. In later years, biochemical methods, in specific, isozyme polymorphism and immunological variability, has helped refine the characterization of pathogens further. Despite the availability of highly sensitive molecular methods of characterization in the present day, biochemical methods remain popular, because of their simplicity, economy and ease of interpretation, for the information that they afford.

The 1964 IUPAC-IUB Commission on Biochemical Nomenclature (CBN) recommended that "multiple enzyme forms" in a single species should be known as isoenzymes (or isozymes). It is known that enzymes catalyzing essentially the same reaction may differ in various ways, as shown in the table below, which includes some prominent examples.

Table 1. Multiple forms of enzymes

Group	Reason of multiplicity	Example
1	Genetically independent proteins ^a	Malate dehydrogenase in mitochondria and cytosol
2	Heteropolymers (hybrids) of two or more polypeptide dehydrogenase chains, noncovalently bound ^a	Hybrid forms of lactate dehydrogenases
3	Genetic variants (allozymes) ^a	Glucose-6-phosphate dehydrogenases in man
4	Conjugated or derived proteins a. Proteins conjugated with other groups b. Proteins derived from single polypeptide chains	a. Phosphorylase b, glycogen synthase b. The family of chymotrypsins arising from chymotrypsinogen
5	Polymers of a single subunit	Glutamate dehydrogenase of molecular weight 1,000,000 and 250,000
6	Conformationally different forms	All allosteric modifications of enzymes

^a These classes fall into the category of isozymes

Isozymes were first described by Hunter and Markert (1957) who defined them as different variants of the same enzyme having identical functions and present in the same individual. This definition encompasses (1) enzyme variants that are the product of different genes and thus represent different loci (described as *isozymes*) and (2) enzymes that are the product of different alleles of the same gene (described as *allozymes*).

Isozymes are enzymes that differ in amino acid sequence but catalyze the same chemical reaction. They usually display different kinetic parameters (i.e. different K_M values), or different regulatory properties. Isozymes are usually the result of gene duplication, but can also arise from polyploidisation or hybridization.

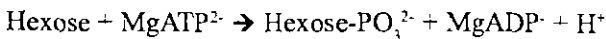
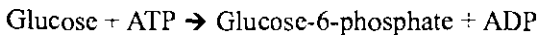
- Over evolutionary time, if the function of the new variant remains *identical* to the original, then it is likely that one or the other will be lost as mutations accumulate, resulting in a pseudogene.
- However, if the mutations do not immediately prevent the enzyme from functioning, but instead modify either its function, or its pattern

of gene expression, then the two variants may both be favoured by natural selection and become specialised to different functions. For example, they may be expressed at different stages of development or in different tissues.

Allozymes may result from point mutations or from insertion-deletion (*indel*) events that affect the DNA coding sequence of the gene. As with any other new mutation, there are three things that may happen to a new allozyme:

- It is most likely that the new allele will be non-functional, resulting in low fitness and therefore removal from the population by natural selection.
- Alternatively, if the amino acid residue that is changed is in a relatively unimportant part of the enzyme, for example a long way from the active site then the mutation may be selectively neutral and subject to genetic drift.
- In rare cases the mutation may result in an enzyme that is more efficient, or one that can catalyse a slightly different chemical reaction, in which case the mutation may cause an increase in fitness, and be favoured by natural selection.

An example of an isozyme is **glucokinase**, a variant of **hexokinase** which is not inhibited by glucose 6-phosphate.



Its different regulatory features and lower affinity for glucose (compared to other hexokinases), allows it to serve different functions in cells of specific organs, such as control of insulin release by the beta cells of the pancreas, or initiation of glycogen synthesis by liver cells. Both of these processes must only occur when glucose is abundant, or problems occur.

Distinguishing isozymes

Isozymes (and allozymes) being variants of the same enzyme, the differences in their biochemical properties (for example their substrates and enzyme kinetics) are too subtle to aid in their being distinguished by a biochemical assay. Isozymes may differ in other ways, in particular, amino acid substitutions that change the electric charge of the enzyme (such as replacing aspartic acid with glutamic acid), which are simple to identify by gel electrophoresis, and this forms the basis for the use of isozymes as

molecular markers. To identify isozymes, a crude protein extract is made by grinding animal or plant tissue with an extraction buffer, and the components of extract are separated according to their charge by gel electrophoresis. Historically, this has usually been done using gels made from potato starch, however, acrylamide gels provide better resolution, and cellulose acetate gels are now (as of 2005) the norm.

All the proteins from the tissue are present in the gel, so that individual enzymes must be identified using an assay that links their function to a staining reaction, elaborated later. This assay method requires the enzymes to be still functional after separation (native gel electrophoresis), and provides the greatest challenge to using isozymes as a laboratory technique.

Isozymes and allozymes as molecular markers

Despite the amazing diversity seen in life, living organisms also share similarities. Different species of life have evolved over time through a process called speciation. Population genetics is essentially a study of the causes and effects of genetic variation within and between populations, and in the past isozymes have been amongst the most widely used molecular markers for this purpose. Though they have now been largely superseded by more informative DNA-based approaches (such as direct DNA sequencing, single nucleotide polymorphisms and microsatellites), they are still amongst the quickest and cheapest marker systems, and remain an excellent technique for identifying low levels of genetic variation, e.g. quantifying mating systems.

Many basic and applied aspects of plant pathology – usually fungus, nematodes and bacteria - can be studied with isozyme analysis. Isozyme analysis is frequently used for taxonomic purposes for the separation or combination of species, separation of subspecies varieties and intersterility groups.

Electrophoretic banding patterns of isozymes can be interpreted in terms of the alleles and loci that code for the polypeptides. Statistical methods have been developed for population genetics, numerical taxonomy and cladistics (i.e., systematic based on phylogenetic relationships) to express relatedness among samples. Each band on a gel is assigned a descriptive value based either on the net migration of the band from the origin (R_m value) or its position relative to that of the band coded by the most common allele. If no genetic interpretation is planned, each pair of isolates can be compared using simple matching coefficients. The data can be expressed in terms of genetic similarity (closeness of relationship of two individuals or

populations) or genetic distance (dissimilarity between two individuals or populations). Similarity values vary from 0 (most distantly related) to 1 (most closely related). Similarity coefficients are calculated using the formula:

$$\frac{2 \times \text{Bands in comon}}{\text{Bands in isolate A} + \text{Bands in isolate B}} \times 100$$

Matching, similarity and distance coefficients can then be subjected to cluster analysis using multivariate analysis or other clustering procedures to group together the different individuals or populations that resemble each other and to identify causes of variability (like geographic location, subspecific groupings, etc.) Of the several cluster analysis programs available, the two most common are single-linkage cluster analysis and unweighed pair group mean average (UPGMA) cluster analysis. The relationships of individual isolates of entire populations can be summarized in the form of clusters or dendrogrms.

Electrophoresis for enzyme purification

Electrophoresis is a technique in which molecules (enzymes, proteins, amino acids, nucleotides and nucleic acids) are separated by differences in their net charge in the presence of an externally applied electric field, polyacrylamide gel electrophoresis (PAGE) being the most common. Polyacrylamide is a polymer of acrylamide ($-\text{CH}_2\text{CHCONH}_2-$) and methylene bisacrylamide ($-\text{CH}_2\text{CHCONHCH}_2\text{NHCOCH}_2-$) and when prepared as a gel is transparent, thermostable, non-ionic and extremely regular in structure. Polymerization is initiated by the free radicals formed by TEMED (N,N,N,N – tetramethylethylenediamine) in the presence of either ammonium persulfate or riboflavin (and light). As TEMED is a free base, polymerization may be delayed or even prevented at low pH. The gel may be either in the form of a rod or a slab, although the latter is preferred.

Effective pore size of polyacrylamide gels depends on the acrylamide concentration, the pore size decreasing with increase in acrylamide concentrations. Two parameters - %T and %C – describe the composition of a polyacrylamide gel. %T is the total concentration of monomer used (acrylamide + bisacrylamide) in grams per 100 ml and %C is the percentage (by weight) of the total monomer which is the cross linking agent.

Acrylamide is a neurotoxin and needs to be handled using Good Laboratory Practices (GLP) to avoid poisoning. Polyacrylamide is not toxic,

but unpolymerized acrylamide can be present in the polymerized acrylamide and so should be handled with caution.

Dissociating and non-dissociating (native) protein electrophoresis

Proteins run on PAGE in the absence of SDS (sodium dodecyl sulfate, the most common dissociating ionic detergent used) will separate on the basis of their charge to mass ratio. While native (non-denaturing) PAGE does not provide direct measurement of molecular weight, the technique can provide useful information such as protein charge or subunit composition. Native PAGE also has the potential for separating proteins of identical molecular weight, which cannot be resolved with SDS-PAGE (denaturing). In addition, proteins on native PAGE usually retain their activity. This allows enzymes to be detected by sensitive and specific activity stains and delicate proteins to be resolved and recovered in a biologically active form.

The interpretation of native gels is more complex than the interpretation of SDS - PAGE gels. Not only can differences in relative mobility reflect differences in charge, mass or both, but also, proteins may have a pI at or above the pH of the buffer, in which case they will not migrate or will "retro-phorese" backward into the upper buffer chamber.

The equation governing protein mobility in native gels is: $\text{Log Rf} = \log (Y_0) - KRT$

RF - relative mobility, normalized to the dye front or some other standard.

Y₀ - relative mobility of the protein in the absence of any sieving matrix.

R - "retardation coefficient," the extent to which the gel matrix affects mobility.

T - % monomer of the gel matrix.

In the presence of SDS, all proteins have the same Y₀, so that a simple relationship exists between Rf and KR at any given T. In other words, SDS treated proteins, having identical charge to mass ratio, migrate at the same speed in free solution under electric force. With such proteins, if you know the mechanical resistance exerted by the gel, you can determine the mobility. This mechanical resistance, KR, is directly related to molecular weight, so that a determination of KR allows calculation of molecular weight. In native gels, the situation is more complicated. Both Y₀ and KR can vary between proteins. Y₀ is related to the charge, while KR varies with the mass.

Continuous and discontinuous buffer systems

Electrophoresis systems where the same buffer ions are present throughout the sample, gel and electrode vessel reservoirs (even if at different concentrations) at constant pH, are called continuous buffer systems. In contrast, discontinuous (multiphasic) buffer systems employ different buffer ions in the gel compared to the electrode reservoirs, most also include differences in buffer composition and pH. Here a large pore 'stacking' gel is polymerized on top of the smaller pore resolving gel.

Preparation and electrophoresis of polyacrylamide gels

As the topic is on characterization of pathogens using isozymes as markers, the technique for native discontinuous electrophoresis alone is given below.

Stock solutions

- ❖ Acrylamide-bisacrylamide (30:0.8): Dissolve 30 g acrylamide and 0.8 g of bisacrylamide in a total volume of 100 ml water. Filter through Whatman No. 1 and store at 4°C in a dark bottle. Stable for 1-2 months.
- ❖ TEMED: Stable as undiluted solution at 4°C in a dark bottle.
- ❖ Ammonium persulfate (1.5% w/v): Prepare fresh.
- ❖ Riboflavin (0.004% w/v): Solution is stable when stored at 4°C in a dark bottle.
- ❖ Electrophoresis buffers: For high pH discontinuous (Stacks at pH 8.3, separates at pH 9.5)
 - Stacking gel buffer: Tris – HCl (pH 6.8): Dissolve 6.0 g of Tris in 40 ml water and titrate to pH 6.8 with 1 M HCl (~ 48 ml). Adjust to 100 ml final volume.
 - Resolving gel buffer: Tris – HCl (pH 8.8): Dissolve 36.3 g of Tris in 48.0 ml of 1 M HCl and bring to 100 ml final volume with water. Titrate to pH 8.8 with HCl if necessary.
 - Reservoir buffer: Tris – Glycine (pH 8.3) at correct concentration for use: Dissolve 3.0 g Tris and 14.4 g Glycine in water and bring to 1 litre final volume.

Preparation of slab gels

The gel plates are perfectly cleaned to ensure good gel adhesion (by soaking in chromic acid, rinsing off the acid and wiping with ethanol and

acetone before air-drying). In the discontinuous buffer system, the stacking gel is polymerized on top of the resolving gel, the sample wells are formed in the stacking gel. The resolving gel of appropriate concentration is poured first into the space between the glass plates, and allowed to polymerize with a flat meniscus by layering resolving buffer over the gel before it sets. The gel mixture is prepared as per the recipe above and before the addition of TEMED, the solution is degassed using a water pump, to prevent bubble formation and for reproducibility in polymerization rates. The gel may be sealed and stored in a refrigerator to be used the next day or used immediately after pouring the stacking gel.

The resolving gel buffer layered over the polymerized resolving gel is poured off and washed with a small volume of stacking gel buffer. The stacking gel of appropriate concentration is prepared and poured over the resolving gel. A comb is inserted into the solution carefully to avoid trapping air bubbles and left undisturbed to polymerize. For riboflavin-catalyzed polymerization, a fluorescent lamp is placed within 2-5 cm of the gel. After polymerization, the comb is removed carefully and the wells formed for sample loading is cleaned with a syringe needle without damaging the gel and filled with stacking gel buffer and used immediately.

Sample preparation for native protein electrophoresis

Samples to be run on native gels should be prepared in a way, which minimizes denaturation of the proteins. Avoid heat, strong detergents, foaming, over-dilution and minimize the activity of endogenous proteases. Keeping the sample cold and including protease inhibitors should help. The choice of buffer is dictated by the requirements of the protein of interest, although some general principles apply. Isotonic (100 - 150mM salt) buffers of pH 6.5 - 8.5 are best for most applications. Large deviations from this range may destabilize proteins, and will also introduce artifacts into the electrophoresis results. Tris or phosphate buffers work well in this pH range.

Electrophoresis

The glass plates are clamped to the electrophoresis apparatus with the notched side of the glass plate aligned with the notch in the upper reservoir. Reservoir buffer is added to the lower reservoir avoiding air bubbles sticking to the bottom of the gel, after which the sample wells and the upper reservoir is also filled. The samples are loaded carefully to the wells and as it is denser it will sink to the bottom of the well. The anode (-) is connected to the bottom and the cathode (+) to the upper reservoirs.

Electrophoresis is conducted at either constant current or constant voltage, though latter gives constant protein mobility during electrophoresis. Too high current may risk over heating and too low voltage increases electrophoresis time and decreases band resolution due to diffusion. In general slab gels may be electrophoresed during the day at 25 – 30 mA constant current or by stacking at 120 V followed by resolving at 200 V constant voltage.

Analysis of gels after electrophoresis

Slab gels are easily recovered by removing the side spacers and gently levering the glass plates apart at the end away from the notch to avoid damaging the fragile notched end. The resolving gel may be carefully transferred into a tray for soaking in buffers or stains. If enzyme activity is to be detected after gel electrophoresis, care should be taken to minimize loss in activity.

Activity stains are specific for a given enzyme or family of enzymes. For example, detection can be based on the localised precipitation of soluble indicator dyes such as tetrazolium salts which become insoluble when they are reduced by cofactors such as NAD or NADP, which is generated in zones of enzyme activity. Examples of some well defined, commonly used activity stains are given below:

Horseradish peroxidase catalyzes the oxidation of a wide range of substrates, transferring electrons from these substrates to H_2O_2 to produce water. Diamino Benzidine (DAB), when oxidized by HRP, produces an insoluble brown precipitate. Gels soaked in H_2O_2 and DAB will show brown bands over sites of peroxidase activity.

This assay can be adapted to detect a number of other enzymes. If H_2O_2 is left out of the buffer, and peroxidase is added at 50 $\mu\text{g/ml}$, the system will detect H_2O_2 . Inclusion of amino acids allows detection of the H_2O_2 producing enzyme amino acid oxidase. Similarly, addition of an amine will permit detection of amine oxidase, which also releases H_2O_2 . Inclusion of both H_2O_2 and HRP in the assay mix will turn the entire gel brown, except where catalase is present, as achromatic zones.

Another versatile stain is built around the reduction of Nitro Blue Tetrazolium (NBT) to an insoluble blue formazan. Diaphorases which reduce NBT at the expense of NAD(P)H, can be detected by soaking gels in 50mM sodium phosphate, pH 7.8 + NBT + NAD(P)H. This reduction can also be accomplished by superoxide. O_2^- producing enzymes can thus be detected, and superoxide dismutase (SOD), which removes superoxide, may be detected as an achromatic zone on uniformly stained NBT/ O_2^- gels.

Fixing is the process whereby proteins are denatured and precipitated in large insoluble aggregates within the gel matrix, preventing the diffusion of proteins, thus keeping the protein bands sharp and resolved during the staining process. The most commonly used fixatives are solutions of short chain alcohols and acetic acid in water - soaking for 1 hr in 45% methanol, 45% water, and 10% glacial acetic acid - is quite effective. The combination of low pH and high organic solvent content disrupts the hydrogen bonding which holds protein structures together, and exposes hydrophobic portions of the protein core. The result is an uncoiling of the peptide chain, followed by an essentially irreversible association between chains, producing a high molecular weight complex which is trapped inside the gel.

Selected references

- Crawford D J 1989 In: *Isozymes in Plant Biology*. Dioscorides, Portland, Oregon.
- Hames B D & Rickwood D 1994 In: *Gel Electrophoresis of Proteins: A Practical Approach*. II edition. Pp. 31-37.
- Hamrick J L & Godt M J W 1990 In: *Plant Population Genetics, Breeding, and Genetic Resources*. Sinauer, Sunderland http://nationaldiagnostics.com/articles.php/tPath/1_4
- Hunter R L & Merkert C L 1957 *Science* 125: 1294-1295.
- Weeden N F & Wendel J F 1990 In: *Isozymes in plant biology*. Chapman and Hall, London.

16

Molecular approaches for studying the genetic diversity of fungal pathogens and biocontrol agents

M Anandaraj & A Kumar

The culture dependent morphological, physiological and biochemical techniques for taxonomic studies and diversity analysis are laborious, time consuming, and somewhat variable and provide insufficient taxonomic resolution. In contrast, culture independent molecular methods are universally applicable. Two important technical advances have stimulated the use of molecular techniques. Firstly, the advent of PCR has allowed the analysis of small numbers of fungal cells or even single spores, dried herbarium material or extinct organisms. Secondly, the selection of universal oligonucleotide primers specific to fungi has provided easy access to nucleotide sequences. The aim of molecular studies in biodiversity is fourfold: (i) phylogenetic studies, (ii) taxonomic studies, (iii) diagnostic applications and (iv) epidemiology and population genetics.

One of the groups of genes which are most frequently targeted for fungal identification and phylogenetic studies is the one that codes for rRNA. The main reasons for the popularity of rDNA are that it is a multiple-copy, non-protein coding gene, whose repeated copies in tandem are homogenized by concerted evolution, and it is therefore almost always treated as a single-locus gene. Furthermore, ribosomes are present in all organisms, with a common evolutionary origin. Parts of the molecule are highly conserved and serve as reference points for evolutionary divergence studies. The conserved regions alternate with variable regions or divergent domains. The 5.8S, 18S, and 25S rDNAs are transcribed as a 35S to 40S precursor, along with internal and external transcribed spacers (ITS and ETS). All spacers are spliced out of the transcript. Between each cluster is a non-transcribed or intergenic spacer (NTS or IGS) that serves to separate the repeats from one another on the chromosome. The 5S gene takes a variable position and is transcribed in the opposite direction. The total length of one DNA repeat is between 7.7 and 24 kb. Comparisons of the 18S (also called the small-subunit [SSU]) rRNA sequences have been performed to assess the relationships of the major groups of living organisms. The ITS regions are much more variable, but sequences can be aligned with confidence only between closely related taxa. These regions are generally used for species differentiation but may also demonstrate patterns of microevolution. A relative hybridization value of over 80% is generally regarded as indicating

membership in the same species, whereas values of less than 20% are proof of nonidentity. Intermediate values have increasingly been found recently, and these probably indicate subspecific entities.

Protein based markers: Isozymes

Isozymes are alternative enzyme forms encoded by different alleles at the same locus, which can be used as informative genetic markers. Multiple polymorphic loci can be surveyed and typically 2 or 3 are detected at each locus. Randomly sampled Isozymes loci are generally accepted to be of independent genetic origin. However, Isozyme variations in many pathogenic fungi are low or Non-existent.

DNA based markers

RFLP: These are the first DNA based markers developed. They may be the result of length mutation and /or point mutation at a restriction digests of genomic DNA, cDNA or mitochondrial DNA fragments from specific DNA segments amplified using PCR. Thus, depending on the probe used, RFLPs can be used to analyze mtDNA variation, ribosomal (r)DNA region variation, repetitive and single-copy sequence variations. RFLPs are co-dominant markers. This makes them suitable for population genetic studies as well as for linkage map construction. By employing probes that detect multiple loci and dispersed repetitive sequences, the sensitivity of the RFLP can be enhanced to fingerprinting resolution.

AFLP: RFLPs can be converted to AFLPs by ligating adaptors for PCR amplification. The method offers the potential to detect large numbers of amplification products. Although this method does not target specific areas of the genome for marker identification, the large number of loci that can be analyzed in a single experiment greatly improves the chance of identifying markers linked to the chosen locus

PCR with arbitrary primers

Direct Amplification Fingerprinting (DAF) (Cactano- Anolles *et al.* 1991) involves the use of short primers, five to eight nucleotides long, with low or high stringency annealing steps and a two-temperature instead of standard three temperature cycling program. Resulting fragments are separated on polyacrylamide gels and visualized by silver staining. AP-PCR (Welsh & McClelland 1990) is performed with oligonucleotides with 20 or more nucleotides were used as primers. Two cycles with low stringency (allowing for mismatches) were followed by 3 to 40 cycles with high stringency. This method is employed to assay variation "within species"(Williams *et al.* 1990), in which arbitrary short oligonucleotide primers, target unknown sequences in the genome, are used to generate amplification products that often show size polymorphism within species.

RAPD analysis offers the possibility of creating polymorphisms without any prior knowledge of the DNA sequences of the organism investigated. The patterns produced are highly polymorphic, allowing discrimination between isolates of a species if sufficient numbers of primers are screened. RAPDs data can contain artifacts and are not fully reproducible. However, RAPDs have been used to generate quickly and cheaply large numbers of genetic markers useful for linkage mapping. RFLP analysis is a laborious and time-consuming procedure that requires previous information of the genome. All these limitations can be overcome by RAPD analysis. RAPD is useful in generating large numbers of DNA markers for species whose genomes are relatively unknown. RAPD fragments can be used to clone and sequence data, to design PCR primers specific for particular organism which then can function as sequence tagged sites (STSs). This method eliminates the problems associated with reproducibility associated with RAPD analysis.

Primer screening

Primer screening is performed to ascertain the suitability of primers and to select primers for the RAPD analysis. For screening primers PCR reactions are set up using DNA from model organism as template.

PCR with template DNA as variable

For the RAPD analysis, the primers giving positive amplification during screening are selected. These selected primers are used to amplify DNA from test organism. In this reaction, the template DNA is a variable while all other reaction constituents are kept constant.

Post PCR analysis of data

Resolve the PCR products in 1.4% agarose gel using protocols mentioned earlier and record the results for analysis using suitable programme for data interpretation.

Molecular approaches for analyzing the genetic diversity of *Phytophthora capsici* infesting black pepper

M Anandaraj, Sheji Chandran & A Kumar

All the available keys on the taxonomy of *Phytophthora* are traditionally based on morphological, physiological and pathogenic characters. There are always inconsistencies in these characters and overlap of phenotypic characters among species. The advancements in molecular techniques has resulted in the use of DNA- based techniques such as analysis of sequences in the internal transcribed spacer region (ITS) region of the nuclear ribosomal RNA (rRNA) gene cluster and mitochondrial DNA. Analysis of the ITS region has been used to determine interspecific variation and sequence differences have been used to distinguish between *Phytophthora* species and also to study the phylogenetic relationships. Sequencing of rDNA gene clusters provide a relatively rapid identification procedure and enables to distinguish isolates that had previously been identified by morphological methods. It was emphasized by Cooke *et al.* (2000) that the six taxonomic groupings of Stamps *et al.* (1990) do not represent natural assemblages because individual ITS clades contained taxa from multiple Waterhouse groups. Similarly, Forster *et al.* (2000), based on ITS1 sequence analysis, concluded that the morphological characters used in *Phytophthora* taxonomy are of limited value in deducing phylogenetic relationships because they exhibit convergent evolution. ITS sequence analysis involves various steps including molecular cloning. Cloning of any DNA sequence involves four steps namely, amplification, ligation, transformation, and screening/selection. Initially, the DNA fragment of interest needs to be amplified (many copies need to be produced). Amplification is commonly achieved by means of PCR. Subsequently, a ligation procedure is employed whereby the amplified fragment is inserted into a vector. The vector (which is frequently circular) is linearised by means of restriction enzymes, and incubated with the fragment of interest under appropriate conditions that allow for ligation. Following ligation the vector

with the insert of interest is transformed to cells. Finally, the transformed cells are cultured. Modern cloning vectors include selectable antibiotic resistance markers, which allow only for cells in which the vector has been transformed to grow. Further confirmation can be accomplished by means of blue/white screening (λ -factor complementation) on X-gal medium and/or PCR, possibly followed by DNA sequencing.

The basic steps in a gene cloning experiment are as follows.

- PCR amplification of gene of interest and elution of the PCR product from the agarose gel.
- Preparation of vector DNA by cleaving with appropriate restriction enzyme.
- Ligation of digested vector with foreign DNA having termini compatible with those of vector.
- Transformation of ligated vector into an appropriate bacterial host.
- Screening and identification of recombinant vector containing particular insert.

Culturing of *P. capsici*

To ensure the genetic purity the single zoospore cultures are plated on the *Phytophthora* specific medium (PVPH incorporated Carrot Agar) and after 48 hours the mycelial disc is transferred into the carrot agar medium and after the period of incubation of 24-48 hrs the mycelial disc is aseptically transferred into the liquid medium (GYP)

PCR amplification of ITS region in *Phytophthora*

The entire ITS regions encompassing ITS 1, 5.8S and ITS2 of the nuclear rDNA could be amplified using the primers ITS-6 and ITS-4 that produces an amplicon of approximately 900bp. The primer sequences for ITS 6 and ITS 4 are as follows.

ITS 6 5' GAAGGTGAAGTCGTAACAAGG- 3' and

ITS 4 5' TCC TCC GCT TAT TGA TAT GC- 3'.

RFLP PCR

ITS6 and ITS4 were used to identify different species of *Phytophthora*.

The PCR mixture contained

DNA	1.0 μ l (50ng)
Taq pol (1U)	0.5 μ l
Reaction buffer (1X)	2.5 μ l
MgCl ₂ (2 μ M)	3.0 μ l
dNTP (100 μ M)	2.0 μ l
Primes ITS4 and ITS6	1.25 μ l +1.25 μ l (each primer)
Water to makeup	25 μ l

The PCR machine is programmed as follows.

Initial denaturation at 94°C for 3 minutes, 40 cycles of denaturation at 94°C for 1 minute, followed by annealing at 55°C for 1 minute and polymerization at 72°C for 2 minutes and final extension at 72°C for 10 minutes

Restriction digestion of the amplified product

Once the reaction is over, out of 25ml, 5 ml is used to check out the presence of the amplified product of 862bp by agarose gel electrophoresis of 1.4%. Once the presence of the product is confirmed, out of the remaining 20ml was separated into 10 ml each, and the first 10ml is subjected to *Msp* I restriction digestion by adding

0.5 μ l of restriction enzyme (*Msp* I or *Taq* I)

0.2 μ l of restriction enhancer (BSA),

2 μ l of 10x restriction buffer and

7.3 μ l of sterile distilled water, making up the total volume of 20 μ l.

This was subjected to incubation at 37°C for 2 hrs.

The same is done with the left out 10ml of the PCR product but with the change in the restriction enzyme, i.e instead of *Msp* I, *Taq* I (restriction enzyme) is used and incubated at 65°C for 2 hrs.

And the restricted product of *Msp* I and *Taq* I are electrophoresed in 2.4% agarose gel, and is documented.

Selected references

Ausubel F M, Brent R, Kingston R E, Moore D D, Seichmann S G, Smith J A & Sruth K 1995 *Current Protocols in Molecular Biology*, John Wiley and Sons Inc. Volume1.

Caetano-Anolles G, Bassam B J & Gresshoff P M 1991 *Plant Mol. Biol. Rep.* 9 : 294-307.

- Clark J M 1988 *Nucl. Acids Res.* 16 : 9677-86.
- Cohen S N, Chang A C Y & Hsu L 1972 *Proc. Natl. Acad. Sci.* 69 : 2110.
- Cooke D E L, Duncan J M, Wagels G & Brasier C M 2000 *Fungal Genet. Biol.* 30 : 17-32.
- Forster H, Cummings M P & Coffey M D 2000 *Mycol. Res.* 104 : 1055-1061.
- Kaufman D L & Evans G A 1990 *BioTechniques* 9 : 304-6.
- Mezei L M & Storts D R 1994 In: *PCR Technology Current Innovations*. CRC Press, pp. 21-7.
- Oishi M & Cosloy S D 1972 *Biochem. Biophys. Res. Commun.* 49 : 1568.
- Sambrook J, Fritsch E F & Maniatis T 1989. In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Lab. Cold spring harbor, NY.
- Stamps D J, Waterhouse G M, Newhook F J & Hall G F 1990 *Agric. Bur. Inst. Mycol. Pap.* 162.28pp.
- Welsh J & McClelland M 1990 *Nucl. Acids Res.* 18 : 7213-7218.
- Williams J G K, Kubelik A R, Livak K J, Rafalski J A & Tingey S V 1990 *Nucl Acids Res.* 18 : 6231-6235.

Molecular characterization of bacteria

A Kumar, V Vinod & R Aravind

The identification and classification of symbiotic and pathogenic plant-associated bacteria are important both in terms of their agricultural applications, as well as for basic studies on plant-microbe interactions. A number of different phenotypic and genotypic methods are presently being employed for microbial identification and classification. Each of these methods permits a certain level of phylogenetic classification, from the genus, species, subspecies, biovar to the strain specific level. Moreover, each method has its advantages and disadvantages, with regard to ease of application, reproducibility, requirement for equipment and level of resolution (Akkermans *et al.* 1995).

A variety of phenotypic methods has been traditionally used to type bacteria, including serotyping, phage typing, microscopic identification, substrate utilization screening (*e.g.* BIOLOG), multilocus enzyme electrophoresis (MLEE), fatty acid methyl ester analysis (FAME), 2-D PAGE of total proteins, and intrinsic antibiotic resistance profiling. Majority of these techniques requires purification and cultivation of the bacteria or can be quite laborious and time consuming (Hook *et al.* 1991). The resulting taxonomy, however, does not necessarily reflect phylogeny, relationships by evolutionary descent. Most microbiologists would prefer to have taxonomic schemes based on phylogeny since the grouped bacteria should share close genetic backgrounds and thus common phenotypes. Phylogenetic analysis can be based on the amino acid sequence of proteins and on the presence of similar metabolic pathways (two early methods), but the most accurate method of determining phylogenetic relationships is the comparison of DNA composition and sequence.

1. Restriction Fragment-Length Polymorphism (RFLP)

With the discovery of restriction endonucleases, rapid methods were devised to identify and cluster bacteria by what has been called Restriction Endonuclease Analysis (REA) and Restriction Fragment-Length Polymorphism (RFLP) analysis. Genomic DNA from most bacteria when digested with "6-basepair cutters" like *EcoRI*, produces about 1000 DNA

fragments of varying sizes that can be analyzed (separated) by agarose gel electrophoresis. The collection of restriction endonuclease-generated DNA fragments from a given species or strain of bacteria gives a distinctive pattern when analyzed by gel electrophoresis, and this pattern can be used to differentiate bacteria or strains of bacteria. This whole-genomic-pattern method is but one example of REA. RFLP analysis is a technique used mainly to detect genetic variation in a single gene. A polymorphism (difference in fragment size) of a specific restriction endonuclease fragment has to be "linked" to a specific genetic allele. The fragment size is monitored by gel electrophoresis, and the specific fragment is usually identified by Southern blotting and probing the DNA pattern with a labeled DNA fragment that hybridizes only to the one, specific fragment. The shortcomings of both REA and RFLP analysis is that they again are slow labor intensive and usually require a large amount of DNA.

2. Random Amplification of Polymorphic DNA (RAPD)

RAPD analysis is a PCR based molecular marker technique. Single short oligonucleotide primers are arbitrarily selected to amplify a set of DNA segments distributed randomly throughout the genome. The DNA amplification product is generated from a region which is flanked by a part of 10 bp priming sites in the appropriate orientation. Genomic DNA from two different individuals often produces different amplification patterns. A particular fragment generated for one individual but not for other represents DNA polymorphism and can be used as a genetic marker.

Interpretations

- ✓ RAPDs exhibit polymorphism and thus can be used as genetic markers.
- ✓ RAPDs are dominant in the sense that the presence of a RAPD band does not allow distinction between hetero- and homozygous states.
- ✓ RAPD markers linked to genes of interest can serve as starting points for chromosome walk to isolate those genes.
- ✓ RAPD protocols are relatively simple.
- ✓ In some cases, RAPD bands of differing lengths can be assigned to the same locus. In that case, these RAPD bands are codominant

3. REP-PCR

Versalovic *et al.* (1994) described a method for fingerprinting bacterial genomes by examining strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes. Two main sets of repetitive elements are used for typing purposes. The repetitive extragenic palindromic (REP) elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem (Stern *et al.* 1984.) REP sequences have been described for numerous enteric bacteria (Gilson *et al.* 1984; Hulton *et al.* 1991). The palindromic nature of the REP elements and their ability to form stem-loop structures have led to multiple proposed functions for these highly conserved dispersed elements (Gilson *et al.* 1990; Yang and Ames 1988).

Rep-PCR can be performed with DNA extracted from bacterial colonies (Woods 1993). Rep-PCR is fast becoming the most widely used method of DNA typing. The technique is easy to perform and can be applied to large or small numbers of isolates. Rep-PCR shows broader species applicability and better discriminatory power than either plasmid profiling or genomic fingerprinting (Georghiou 1995.). Rep-PCR has considerably better discriminatory power than restriction analysis of the 16S rRNA gene or the 16S-23S-spacer region (Appuhamy 1997). Furthermore, studies which have compared Rep-PCR to other typing methods such as multilocus enzyme electrophoresis, biochemical characterizations, or ribotyping (Vila 1996). A comparative study of different PCR-based DNA fingerprinting techniques for typing has shown Rep-PCR to be superior to these methods.

4. ERIC PCR

The Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences are a second set of DNA sequences which have been successfully used for DNA typing. ERIC sequences are 126-bp elements that contain a highly conserved central inverted repeat and are located in extragenic regions of the bacterial genome (Sharples and Lloyd. 1990). They have been defined primarily based on sequence data obtained from *E. coli* and *Salmonella typhimurium*.

5. BOX PCR

While the REP and ERIC sequences are the most commonly used targets for DNA typing, another repetitive element, the BOX sequence, has been used to differentiate bacteria (Koeuth *et al.* 1995). BOX elements are located within intergenic regions and can also form stem-loop structures due to their dyad symmetry. They are mosaic repetitive elements composed

of various combinations of three subunit sequences referred to as boxA, boxB, and boxC (Martin *et al.* 1992) The three-subunit sequences have molecular lengths of 59, 45, and 50 nucleotides, respectively. The BOX elements have no sequence relationship to either REP or ERIC sequences (Martin *et al.* 1992). While initially thought to be unique to *S. pneumonia*, BOX elements have now been found in a number of bacterial species

6. Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The conserved nature of the rRNA sequences has also led to the development of generic DNA probes for bacteria. The amplified ribosomal gene (rDNA) is subjected to restriction endonuclease digestion this has been termed ARDRA (Amplified Ribosomal DNA Restriction Analysis). The resulting restriction fragment pattern is then used as a fingerprint for the identification of bacterial genomes. This method is based on the principle that the restriction sites on the RNA Operon are conserved according to phylogenetic pattern (Woese 1987; Stackebrandt and Goebel 1994). The 16S rRNAs are valuable phylogenetic marker molecules for microorganisms because they are universally distributed and constant in function and because different positions of their sequences change at very different rates. This makes the 16S rRNA gene suitable for many analytical methods, like sequence analysis, ribotyping, restriction fragment length polymorphism (RFLP) analysis, and hybridization with oligonucleotide probes, all useful for the identification and typing of microorganisms. Of all these methods. RFLP analysis of PCR generated rDNA fragments named ARDRA (amplified rDNA restriction analysis) is used very common.

Conclusion

Clearly, the utility of DNA-based approaches has been enhanced tremendously by the application of PCR. A most useful, application of PCR to bacterial identification and classification has been in the area of genomic fingerprinting. Different PCR-based DNA fingerprinting techniques were used for the identification of bacteria. The PCR-based DNA fingerprinting techniques were: (i) repetitive extragenic palindromic (REP) PCR; (ii) enterobacterial repetitive intergenic consensus (ERIC) PCR; (iii) BOX PCR; (iv) randomly amplified polymorphic DNA (RAPD); (v) restriction analysis of the amplified 16S rRNA gene (ARDRA-16S); and (vi) restriction analysis of an amplified region containing the 16S-23S rRNA spacer region and part of the 23S rRNA gene (ARDRA 23S + spacer).

Selected references

- Akkermans A D L, van Elsas J D & de Bruijn F J 1995 *Molecular Microbial Ecology Manual*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1-488.
- Appuhamy S, Parton R, Coote J G and Gibb H A 1997 *J. Clin. Microbiol.* 35: 288-291.
- Georghiou P R, Hamill R J, Wright C E, Versalovic J, Koeuth T D A, Watson & Lupski J R 1995 *Clin. Infect. Dis.* 20: 84-94.
- Gilson E, Perrin D & Hofnung M 1990 *Nucleic Acids Res.* 18: 3941-3952.
- Gilson E, Clement J M, Brutlag D & Hofnung D 1984 *EMBO J.* 3: 1417-1421.
- Hook L A, Odelsen D A, Bogardt A H, Hemmingsen B B, Labeda D P & MacDonell M T 1991 *Newsletter (USFCC)* 21 (3): 1-10.
- Hulton C S J, Higgins C F & Sharp P M 1991 *Mol. Microbiol.* 5: 825-834.
- Koeuth T, Versalovic J & Lupski J R 1995 *Genome Res.* 5: 408-418.
- Martin B, Humbert O, Camara M, Guenzi E, Walker J, Mitchell T, Andrew P, Prudhomme M, Alloing G & Hakenbeck R 1992 *Nucleic Acids Res.* 20: 3479-3483.
- Sharples G J & Lloyd R G 1990 *Nucleic Acids Res.* 18: 6503-6508.
- Stackebrandt E & Goebel B M 1994 *Int. J. Syst. Bacteriol.* 444: 846-849.
- Stern M J, Ames G F L, Smith N H, Robinson E C & Higgins C F 1984 *Cell* 37: 1015-1026.
- Versalovic J, Schneider M, de Bruijn F J & Lupski J R 1994 *Meth. Cell. Mol. Biol.* 5: 25-40.
- Vila J, Marcos M A & Jimenez de Anta M T 1996 *J. Med. Microbiol.* 44: 482-489.
- Woese C R 1987 *Microbial Rev.* 51: 221-271.
- Woods C R, Versalovic J, Koeuth T & Lupski J R 1993 *J. Clin. Microbiol.* 31: 1927-1931.
- Yang Y & Ames G F L 1988 *Proc. Natl. Acad. Sci. USA* 85: 8850-8854.

19

Molecular systematics- basic concepts

K Nirmal Babu & J Martin

The theory of evolution is the foundation upon which much of modern biology is built. Systematics is the scientific study of the kinds and diversity of organisms and relationships among them. It includes taxonomy (identification, nomenclature and classification), process of evolution (study of mechanics of short-term evolution, including sources and organization of variability in populations, the origins of species, and hybridization) and phylogeny (long-term evolution or how different species may have evolved from one another, the divergence or isolation of groups with respect to time and location). Systematics was established as a mainstream scientific field by Carolus Linnaeus (1707-1778). It is a hierarchical system of classification. Groups are nested within larger groups. Levels: kingdom, phylum, class, order, family, genus, species.

The study of the relationships between groups of organisms is called phylogenetics which depends on study of structural, developmental and genetic (DNA, RNA, Protein sequence etc) homology. The subject of phylogenetics was developed by Willi Hennig, a German entomologist, in 1950s and is essentially based on the understanding that groups of similar organisms are descended from a common ancestor and phylogenetic systematics is a method of taxonomic classification based on their evolutionary history.

These evolutionary relationships are documented by creating a branching structure, termed a phylogeny or tree that illustrates the relationships between the characters and also sequences. In general it follows two pathways as given below.

- Cladistic methods to construct a tree (cladogram) by considering the various possible pathways of evolution and choose from among these the best possible tree.
- A phylogram is a tree with branches that are proportional to evolutionary distances.

Molecular evolution

With the advent of molecular genetic methods and the development of conceptual framework for reconstructing phylogenetic trees, systematics has become a rigorous scientific discipline over the past few decades. Molecular data is a powerful tool for evolutionary studies because it is not dependent on cultural conditions, where as morphological and physiological characters often are. It is often easier to quantitate than morphological data. Some organisms have few morphological characters that can be used for taxonomic purposes. Phylogenetics often makes use of numerical data, which can be scored for various "character states" such as the size of a visible structure (numerical taxonomy) or use DNA sequences which is being increasingly available. Similarities and differences between organisms can be coded as a set of characters, each with two or more alternative character states including the alignment of DNA, RNA or protein sequences. The major uses of phylogenetics are determining the closest relatives of the organism that you're interested in, discovering the function of a gene and retracing the origin of a gene.

DNA is a good tool for taxonomy. DNA sequences have many advantages over classical types of taxonomic characters in that character states can be scored unambiguously and information on both the extent and the nature of large numbers of characters can be scored for each individual; Divergence between sequences is mainly because of nucleotide substitutions, insertion/deletions, or genome rearrangements.

Each nucleotide difference is a character. Sequences reflect relationships, among genes and among organisms. The understanding is that for a given gene, closely related organisms have similar sequences and more distantly related organisms have more dissimilar sequences. These differences can be quantified.

Profiling using molecular markers – RAPD, SSR, ISSR, AFLP, SNP, CAPS, DAF etc. and more recently rRNA genes, cytochrome-C genes, mitochondrial DNA sequences, ITS regions have become important tools for phylogenic studies. We have to choose specific sequences to make either a gene tree or a species tree. These sequences may be modified over time due to mutation which reflects the evolutionary process. Similarity searches and multiple alignments of these sequences naturally lead to the question on relatedness of the sequences which may reflect the relatedness of the organisms from which these sequences come.

Species may have similar character states for three reasons. They share a derived character state that originated in a common ancestor. They share an ancestral character state for example, humans and iguanas each have five toes, whereas a horse has one, but we're still closer to horses. They share a feature that is not homologous. Independent evolution of the same character in two separate lineages is called homoplasy. If there were no homoplasy, and all lineages evolved at about the same rate, then overall similarity would work. Relationships can be based on information on homologous genes which are genes that derive from a common ancestor. They can be

Orthologs: which are homologues in different species with analogous functions.

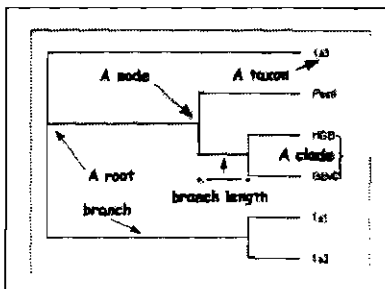
Paralogs: which are homologues separated by a gene duplication (different but related functions).

Xenologs: which results from a lateral transfer between two organisms.

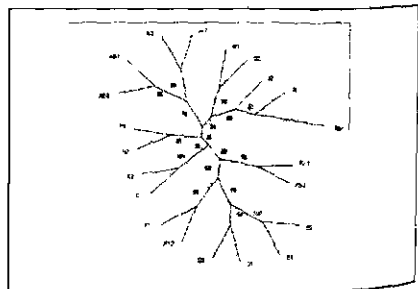
A phylogeny that includes both orthologs and paralogs is likely to be incorrect. Sometimes phylogenetic analysis is the best way to determine if a new gene is an ortholog or paralog to other known genes.

The evolutionary relationships of taxa can be illustrated by a phylogenetic tree where the taxonomic units (species, genes) are represented by the nodes. The relationships of OTUs (operational taxonomic units) are defined by branches.

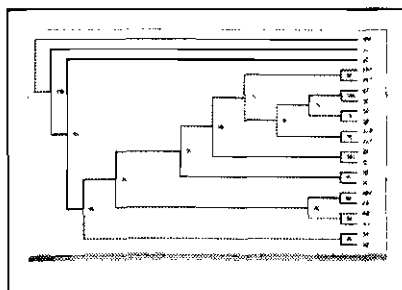
The distances of the branch are referred to the branch length. Phylogenetic tree can be in the form of rooted and unrooted tree.



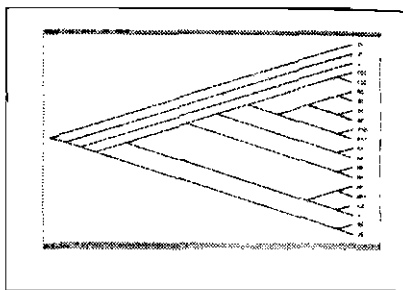
rooted tree



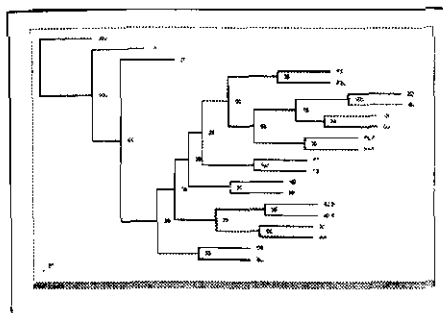
unrooted tree



Rectangular cladogram



Slanted cladogram

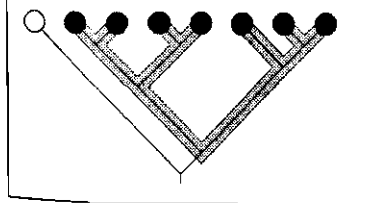


Phylogram

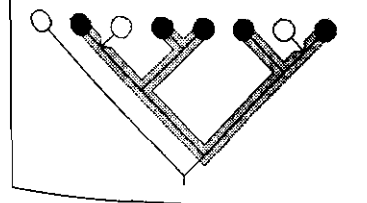
Some considerations should be kept in mind:

Monophyletic taxon : A group composed of a collection of organisms, including the most recent common ancestor of all those organisms and all the descendants of that most recent common ancestor. A monophyletic taxon is also called a clade. Examples: Mammalia, Aves (birds), angiosperms, insects, fungi, etc.

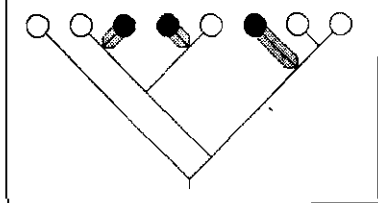
Monophyletic taxon (clade) :



Paraphyletic taxon :



Polyphyletic taxon :



Paraphyletic taxon: A group composed of a collection of organisms, including the most recent common ancestor of all those organisms. Unlike a monophyletic group, a paraphyletic taxon does not include all the descendants of the most recent common ancestor. Examples: Traditionally defined Dinosauria, fish, gymnosperms, invertebrates, protists, etc.

Polyphyletic taxon: A group composed of a collection of organisms in which the most recent common ancestor of all the included organisms is not included, usually because the common ancestor lacks the characteristics of the group. Polyphyletic taxa are considered “unnatural”, and usually are reclassified once they are discovered to be polyphyletic. Examples: marine mammals, bipedal mammals, flying vertebrates, trees, algae, etc.

For systematic classification, only monophyletic groupings describe evolutionarily cohesive units. Phylogenetically informative characters in cladistics as given below.

- ❖ **Synapomorphies:** Shared derived characters are the most useful characters for assessing phylogenetic relationships. They are *homologous* characters that reflect descent from a common ancestor.
- ❖ **Autapomorphies:** Characters that are unique to a single taxon are uninformative for assessing the phylogenetic relationships among groups.
- ❖ **Pleisiomorphies, Symplesiomorphies:** Shared primitive characters are useless for reconstructing the phylogenetic relationships among taxa.

Autapomorphies can become synapomorphies over time.

Other approaches for phylogenetic reconstruction

Parsimony: The shortest tree is the best estimate of the phylogeny (A cladistic method). These techniques maps characters one-by-one, and through tree searches find the shortest overall tree.

Neighbor-joining: A distance-based method. It uses the overall genetic divergence between the taxa, rather than an analysis of individual characters (derived from the phenetic approach).

Maximum likelihood: This method uses a concrete model of the evolutionary process (sequence evolution). It then evaluates, for each possible phylogenetic tree, the likelihood of the observed data. The tree that is most likely to yield the data is the best estimate (A different approach altogether).

Some softwares used for phylogenetic analysis: With so much of molecular data available analysis and interpretations of sequence similarities and

utilizing them to understand phylogeny is impossible without the help of computational software. Some important softwares and the sites where details of each software available are given below.

1. MrBayes Bayesian (DNA or protein): Free, good documentation, Very flexible. Details at <http://morphbank.ebc.uu.se/mrbayes>.
2. PAUP*: Maximum likelihood (DNA only), parsimony, distance, Not free, Very flexible regarding setting parameters, constraining trees. Details at <http://paup.csit.fsu.edu/>
3. Phylip: Maximum likelihood, parsimony, distance (DNA, protein, etc.), Free, Good documentation. Details at <http://evolution.genetics.washington.edu/phylip.html>
4. Other programs: MEGA 3.1 (<http://www.megasoftware.net>), Tree-Puzzle (<http://www.tree-puzzle.de/>), PAML (<http://abacus.gene.ucl.ac.uk/software/paml.html>)

Other useful softwares for molecular systematics

A. Sequence alignment

1. MUSCLE (<http://www.drive5.com/muscle/>)
2. ClustalW

B. Sequence editors

1. SeqLab (available in GCG package on socrates)
2. Jalview (<http://www.jalview.org/>)
3. Bioedit (PC only; <http://www.mbio.ncsu.edu/BioEdit/page2.html>)

C. Format conversion

1. Readseq (<http://www.ebi.ac.uk/cgi-bin/readseq.cgi>)

D. Tree viewing and manipulation

1. Treeview (full versions for PC and Mac OS9; basic version for UNIX/LINUX/Mac OSX: <http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>)
2. TreeExplorer in MEGA3.1 (<http://www.megasoftware.net/>)
3. Hypertree (for large trees; <http://www.kinase.com/tools/HyperTree.html>)

Selected references

- Graur D & Li W H 2000 *Fundamentals of Molecular Evolution*, 2nd Edition. Sinauer Associates.
- Hall B 2004 *Phylogenetic Trees Made Easy: A How-To Manual*, 2nd Edition. Sinauer Associates.
- Li W H 1997 *Molecular Evolution*. Sinauer Associates.
- Nei M & Kumar S 2000 *Molecular Evolution and Phylogenetics*. Oxford University Press.

IV

DETECTION AND DIAGNOSIS

Role of diagnostics in disease management

M Anandaraj

The ever increasing global population exerts pressure on the food production system to produce more in order to meet the growing demand. On the World food day 16 October 2006, Mr. Jacques Diouf, Director General of Food and Agriculture Organizations of United Nations raised concerns about the increasing populations of hungry millions all over the world. There is limited scope to increase the area but there is tremendous scope to reduce the crop losses caused by pests and pathogens which is estimated to be about 40%. Diseases in plants are caused by various organisms such as fungi, bacteria, viruses, phytoplasma, insect pests, nematodes, other herbivores besides parasitic plants.

Diseases caused by microbes are severe and occur in epidemic and pandemic proportions. Epidemiologically significant increase in pathogen populations often goes un-noticed. Once the epidemic is set in motion, it is impossible to check its spread. The initial detection of the potential pathogen plays a vital role in the management of plant epidemics. Diagnostics is the process of confirming the presence of the potential pathogen and this paves the way for other methods of disease management.

Plant disease epidemics

The science of plant pathology has progressed considerably from the time of the description of late blight of potato by the oomycetous pathogen *Phytophthora infestans*. The description of the disease as a triangle consisting of susceptible host, the virulent pathogen and a favourable weather has given way to the tetrahedron by including the influence of man and further to disease polyhedron adding time and space. Diagnosis of human diseases by adopting latest technologies has been progressing faster than that of plant diseases. The mad cow disease and avian flu are the two recent examples where appropriate action has been taken to prevent their spread to humans. Similarly to prevent crop plant diseases several actions have been developed. Diagnostic kits are available to detect microbial contaminants like aflatoxin in processed food and feed and various regulations on the maximum permissible limits.

The need for plant disease diagnostics

The advent of WTO and globalization has brought increased movement of plants and there is increased possibilities of movement their pests and pathogens across political and physical barriers of countries and continents. The international agricultural trade is regulated by many International Standards for Phytosanitary Measures evolved by International Plant Protection Convention (IPPC) to prevent introduction of new pests and pathogens. The sanitary and phytosanitary (SPS) methods prevent the introduction of pests and pathogens that affect human, animal and plant health. With increased free trade among countries and removal trade restrictions at times SPS could be used to restrict the import of goods if exporting countries are not following the standards set by importing countries. There are no separate standards for SPS and already existing standards governing food such as CODEX standards are equalized with SPS. There is an increased risk of misuse of SPS provision to restrict import of goods. Under SPS agreement the WTO may force a nation to lower its health standards or pay international penalty. The penalty could be in the form of compensating the foreign government where exports to the nation are limited by stricter standards. To avoid use of SPS as a guise to trade restriction, the SPS regulations must be based on scientific principles and based on evidences. A nation may challenge another nation's food safety standards being too high. The availability of diagnostic kits would be of help to scientifically assess the damage potential of pathogens and this becomes an essential component in disease detection in the WTO regime.

Pest Risk Analysis (PRA) and plant quarantine

The IPPC defines PRA as 'the process of evaluating biological or other scientific and economic evidence to determine whether a pest should be regulated and strength of any phytosanitary measures to be taken against it'. The objective of PRA are to identify pests and or pathways of quarantine concern, evaluate their risks, identify endangered areas and if appropriate identify management options. The diagnostic methods are used effectively by regulatory authorities in plant quarantine. The recent example is Karnal bunt of wheat. The disease is caused by *Tilletia indica* and was reported from India, Pakistan, Nepal and Afghanistan in 1930. By 1972 it was reported in Mexico and it reached the Arizona coast in United States within a couple of years. It was considered as quarantine pathogen and its further spread arrested by strictly following quarantine method by concerted efforts of plant pathologists, bioinformatics scientists and quarantine officials. The measures included, initial identification of the pathogen, confirming its identity and complete destruction crop by deep ploughing and restricting the movement of seed material from the affected area to other parts of the country. The speed of detection and diagnosis are vital for the arrest of

movement of diseases across geographic areas. Thus the emerging diseases are ever increasing threat for plant pathologists all over the world. The shrinking of world in to a global village and free movement of people poses greater threat of introducing new diseases. The availability of experts and expertise are essential in managing quarantine pests and pathogens. The diagnostic tools and methods are the key components essential for the detection of pathogens that are recorded previously or occurring afresh in a locality.

Ever since the attack on world trade centre and animosities among countries, there is a perceived threat of biological warfare by introduction of plant pathogens deliberately. Crop plants would be a soft target for bio-terrorism and there is an increased need to develop rapid detection and diagnostic methods to prevent any possible biological warfare. Disease detection plays a key role in arresting many new plant disease epidemics.

Diagnosis of plant diseases

A simple diagnosis requires only symptom recognition and a test to confirm the identity. This is for already known disease and requires the skill and expertise of an expert. The presence of databases and the information retrieval system using bioinformatics tools for data mining makes it possible to confirm the identity of previously recorded pathogen any where on the globe. Whereas, to diagnose a hitherto unknown disease needs lot of time and expertise in the conventional diagnosis. There is a need to visit the infected field, record the symptoms, isolate the pathogens and prove the Koch's postulates. The modern tools also enable identification of hitherto unrecorded species by use of information technology. The general tenet of plant disease management is to avoid the disease. The other methods of resisting and living with the disease come later. Disease diagnosis helps to prevent the disease entry and helps in quarantine regulations (Refer Page 164 for a chapter on quarantine regulations)

Identification of diseases based on symptoms is easy but such of the plants without symptoms could not be identified. Isolation of pathogens on to culture media is long and cumbersome and not all pathogens are cultivable. There are various methods available for detection of plant pathogens. Serology based detection and diagnosis of plant viruses has been done since 1960s. Whereas, it has not been very successfully used against other pathogens such as fungi, bacteria and nematodes. Serology based detection of pathogens such as *Phytophthora* has been developed but has not been used extensively. The advancements made in molecular biology has now given us an opportunity to analyze the DNA sequences especially of the internal transcribed spacer (ITS) regions of ribosomal genes. Species specific probes to detect pathogens are being developed that has great potential in detection and diagnostics.

Serological methods

Several serological methods have been developed for detection of plant pathogens. The widely used methods are as follows. The enzyme linked immunosorbent assay (ELISA) is most frequently used for several pathogens; Immuno-fluorescence (IF), colony staining and several variants. Antisera for over 800 pathogens are available for the American Type Culture Collections in the US. Both polyclonal and monoclonal antibodies are reported for detection of several diseases and are available commercially. There are also a number of protocols such as immuno diffusion assay, Western blots, dot blots immunosorbent assays, immunostrip assay and serologically specific electron microscopy (SSEM). (Refer Page 79 and 147 for more details)

PCR based methods

PCR based methods are available for several groups of plant pathogens such as bacteria, fungi and viruses. Both classical and real time fluorescent based PCR (RT-PCR) are available. In Real Time PCR, the principle of fluorescent resonance energy transfer is applied, and the amplified products are detected and are also known as molecular beacons. The principle involves hybridization of fluorescently labeled oligonucleotide probe to a specific region on the target amplicon that is amplified using traditional forward and reverse primers. In Taqman™ system an oligonucleotide probe of 25-30 nucleotides labeled at 5' with fluorochrome such as 6-carboxyfluorocin (6-FAM) and a quencher fluorochrome 6-carboxytetramethyl rhodamine (TAMRA) at 3' end. The two probes are designed in such a way that they hybridize the PCR product they are aligned head to tail to bring the two fluorescent dyes together. The fluorescent dye attached to the first probe is excited by the light source of the appropriate wavelength and emits green fluorescent light at slightly longer wave length. When the second probe is in close proximity, the energy emitted by the first probe excites the light cyclor red 640 attached to the second probe and the light emitted could be detected at 640nm. The Taqman™ probe is degraded by 5' to 3' exonuclease activity of the Taq polymerase as it extends the primer during each PCR amplification cycle and the fluorochrome is released. The amount of fluorescence is monitored and it is proportional to the quantity of PCR product.

Genetic approaches include PCR based methods. Multiplex PCR, Cooperative PCR (CoPCR), nucleic acid sequence based amplification (NASBA) have also been used. Other PCR methods like rep-PCR, ERIC and BOX PCR are also being used to study the variations in populations of several plant pathogens such as *Ralstonia solanacearum*, *Xanthomonas* and *Erwinia*. An integrated approach involving enrichment assays, immunomagnetic separation followed by culturing, fluorescent in situ

hybridization (FISH) and probes specific for r-RNA and RFLP involving 16S – 23 S r DNA are also being used extensively.

Nematodes have been identified using conventional morphological characters involving adults of both the sexes. Recent advances shows that PCR based probes targeting r-RNA genes of 18S, 5.8S and 28S and mitochondrial genome of cytochrome oxidase (CO1) could be used effectively not only for identification but also for population studies.

Conclusion

The availability of diagnostic tests ensures applications in the detection of plant pathogens in the planting materials and aids in the adoption of disease management strategies. Quarantine pests could be identified and precautions taken to prevent its introduction to new areas. The bioinformatics tools available could be used along with PRA diagnostic tools to prevent plant diseases and to evolve viable strategies to prevent crop losses. There is an urgent need to interlink various organizations involved in disease surveillance, pathogen detection and diagnosis, plant quarantine and other agricultural developmental agencies in order to act in unison in case of an exigency of threat to crop plants by pathogens introduced accidentally or intentionally so that India's food security is ensured.

Selected references

- Martin R, James D & Levesque C A 2000 *Ann. Rev. Phytopathol.* 38: 207-239.
- Stewart T M & Galea V J 2006 *Plant Dis.* 90: 539-547.
- Schaad N W, Frederick R D, Shaw J, Schneider W L, Hickson R, Petrillo M D & Luster D G 2003 *Ann. Rev. Phytopathol.* 41: 305-324.
- Waard E, Foster S J, Fraaije B A & McCartney A H 2004 *Ann. Appl. Biol.* 145: 1-16.

21

Applications of Genomics and Proteomics in diagnostics

R Manimekalai

Introduction

Detection of pathogenic bacteria and viruses in plant material, vectors or natural reservoirs is essential to ensure safe and sustainable agriculture. The available techniques, especially those based on molecular techniques, have evolved significantly in the last few years to allow the rapid and reliable detection of pathogens (Rosie *et al.* 2004).

Molecular detection is largely based on PCR or RT-PCR amplification following purification of nucleic acids from the samples, with the extraction of the target DNA. Variants of PCR, such as simple or multiplex nested-PCR in a single closed tube, co-operative-PCR, and real-time monitoring of amplicons or quantitative PCR, allow high sensitivity in the detection of one or several pathogens in a single assay. The latest development is the microarray technology, but it requires generic DNA/RNA extraction and pre-amplification methods to increase detection sensitivity. The advances in genomics and proteomics represent a new source of information for the development of sensitive and specific detection techniques for these microorganisms. It aid in multiplexing, i.e., many pathogens per assay, with faster and highly accurate detection capabilities (Levesque 2001).

Terminologies and technologies in genomics and proteomics

Genomics is the study of an organism's entire genome. Genomics appeared in the 1980s, and accelerated in 1990s with the initiation of genome projects for several species. A major branch of genomics is still concerned with sequencing the genomes of various organisms, although the knowledge of full genomes have created the possibility for the field of functional genomics, mainly concerned with patterns of gene expression during various conditions. The most important tools here are microarrays.

Proteomics is the study of the full set of proteins in a cell type or tissue, and the changes during various conditions, is called proteomics. Proteomics is the large-scale study of protein, particularly their structures and functions. This term was coined to make an analogy with genomics, and while it is

often viewed as the “next step”, proteomics is much more complicated than genomics. Most importantly, while the genome is a rather constant entity, the proteome differs from cell to cell and is constantly changing through its biochemical interactions with the genome and the environment. One organism has radically different protein expression in different parts of its body, in different stages of its life cycle and in different environmental conditions.

The entirety of proteins in existence in an organism throughout its life cycle, or on a smaller scale the entirety of proteins found in a particular cell type under a particular type of stimulation, are referred to as the *proteome of the organism or cell type respectively*. Since proteins play a central role in the life of an organism, proteomics is instrumental in discovery of biomarkers, such as markers that indicate a particular disease.

Key technologies used in proteomics

- i. One- and two-dimensional gel electrophoresis are used to identify the relative mass of a protein and its isoelectric point.
- ii. X-ray crystallography and nuclear magnetic resonance are used to characterize the three-dimensional structure of peptides and proteins.
- iii. Tandem mass spectrometry combined with reverse phase chromatography or 2-D electrophoresis is used to identify (by de novo peptide sequencing) and quantify all the levels of proteins found in cells.
- iv. Mass spectrometry often MALDI-TOF, is used to identify proteins by peptide mass fingerprinting. This technology is also used in so-called “MALDI-TOF MS protein profiling” where samples (i.e. serum) are prepared by either protein chips (SELDI-TOF MS), magnetic beads or with other methods of sample treatment, such as liquid chromatography, size-exclusion and immunoaffinity. Protein peaks of interest must be identified by tandem mass spectrometry. Protein profiling with MALDI-TOF MS could be of high use in clinical diagnostics.

In post-genomics era large number of single-nucleotide polymorphisms (SNPs) have been characterized. This allows the DNA detection to its ultimate limit—the single base pair difference. Likewise, many plant pathogens differ from their closest relatives by only a few bases on different genes.

DNA microarrays, designed to study gene expression or to generate single-nucleotide polymorphisms (SNPs) profiles, are now fabricated with oligonucleotides that are either synthesized directly on a solid surface or that are microspotted. Likewise sequence of the oligonucleotides for all

the plant pathogens can be spotted to make oligonucleotide array. Hence the detection of all plant pathogens are possible at a multiplex level.

The rapid increase in the availability of complete genome sequences and large expressed sequence tag datasets for many plant pathogens propelled the new methods and perspectives to bear the studies of the interactions between plant pathogens and their hosts. The comparison of the completed genomes of hundreds of bacterial genome species, revealing previously intractable evolutionary relationships between these organisms. Similarly, comparing genes present in phylogenetically diverse plant pathogens allows to identify common pathways or strategies used by these organisms and offers the prospect of novel generic control strategies. By the development and use of high-throughput molecular technologies, such as transposon grids and custom microarrays, the processes taking place in pathogens and their hosts to get a complete picture of the processes underlying development of infection is possible, which will design strategies for the detection/control at an early stage of infection.

The researchers at SCRI, UK, completed the genome sequencing of *Erwinia carotovora* subsp. *atroseptica* (*Eca*; the cause of the most economically-significant bacterial disease in UK agriculture). Sequencing whole genome has been essential in the identification of major components of virulence, and in comparison with over 250 other sequenced bacterial genomes, has revealed new insights into the evolution of its ability to cause disease. *Eca* is now one of the subjects of a multi-site Systems Biology research dedicated to unravelling the processes that take place during infection.

The genome sequences of several oomycete plant pathogens are still in the process of being completed. With the available sequence of the pathogen, the common features associated with their ability to cause disease, and unravel evolutionary relationships in the development of this ability can be identified.

Genomics tools are used for studies of the potato cyst nematodes *Globodera rostochiensis* and, particularly, *G. pallida*. Expressed Sequence Tag (EST) analysis and analysis of BAC libraries for examining the biology of the pathogens revealed the horizontal gene transfer from bacteria has played an important role in the evolution of plant parasitism by nematodes.

Applications of molecular methods for the detection of plant pathogens

Real time PCR detection of pathogens

Fabre *et al.* (2003) reported the gel-free real-time one-step reverse transcription PCR (RT-PCR) protocol for specific detection and quantitation

of Barley yellow dwarf virus-PAV, the most widespread BYDV species in Western Europe. The assay is based on TaqMan technology, detects and quantifies from $10(2)$ to $10(8)$ BYDV-PAV RNA copies. They reported that the test is 10^1 and 10^3 times more sensitive than the standard RT-PCR and ELISA assays published previously for BYDV-PAV detection and significantly improves virus detection in single aphids insect.

Multiplex RT PCR

Menzel *et al.* (2002) utilized two multiplex RT-PCR assays with specific coamplification of plant mRNA as an internal control from total nucleic acids for the parallel detection of Apple chlorotic leaf spot virus, Apple stem pitting virus, Apple mosaic virus and Apple stem grooving virus. Four virus specific primer pairs were used for the RT-PCR. They could detect a range of different virus isolates from various geographic origins by these multiplex RT-PCR assays.

Osman *et al.* (2006) described an extraction technique for reverse transcription-PCR (RT-PCR) detection of plant pathogens including viruses, bacteria and phytoplasma. They extracted the total nucleic acid of these plant pathogens by direct spotting of crude sap derived from infected leaf, petiole or cambial tissue onto two different types of membranes, positively charged Hybond N(+) Nylon and FTA membranes. The discs were directly placed in the PCR reaction cocktail. Specific amplification of genomic or ribosomal RNA fragments of these pathogens was obtained by one-step RT-PCR.

Mass spectrometry (MS) -based proteomics for the detection of plant pathogens

MS-based proteomics has become a powerful and increasingly popular approach for identifying the pathogens and to understand their biology. The MS is based on the identifying the pathogen based on its one of the proteins. At present push in the proteomics community to make data from large-scale proteomics experiments publicly available in the form of a centralized repository. Such a resource could enable the use of MS as a universal plant pathogen detection technology (Neerav *et al.* Soybean Genomics and Improvement Laboratory, USDA-ARS)

Analysis of this large-scale genomic data would not be possible without the power of computers, and the genomic research efforts in plant pathogens are aided by the development of custom databases, and visualisation and analysis software.

Conclusion

Recent advances in genomics and proteomics are achieving the multiplexing, speed and portability in pathogen detection. The research in this area has to be strengthened by sequencing sufficient number of plant pathogens and elucidating the mechanism of pathogen evolution. It enables the creation of valuable resources for the comparative genomics of plant pathogens and microarray technology for the diagnostics.

References

- Fabre F, Kervarrec C, Mieuxet L, Riault G, Vialatte A & Jacquot E 2003 Improvement of barley yellow dwarf virus-PAV detection in single aphids using a fluorescent real time RT-PCR. *J. Virol. Methods*. 9:110(1):51-60.
- Levesque CA 2001 Molecular methods for detection of plant pathogens – what is the future? *Can. J. Plant Pathol.* 24: 333-336.
- Menzel W, Jelkmann W and Maiss E 2002 Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. *J Virol Methods* 99: 81-92.
- Neerav DP & Bret C Mass spectrometry-based proteomics for the detection of plant pathogens. Soybean Genomics and Improvement Laboratory, USDA-ARS, Beltsville, MD, USA.
- Osman F & Rowhani A 2006 Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). *J Virol Methods* 133(2):130-6
- Rosie E B, Simon J F & Brendon J M 2004 Molecular diagnostic tools for detection of plant pathogenic fungi. In. *Molecular Diagnostics: Current Technology and Applications*.

Serology and immunodiagnosis of plant pathogens

M Krishna Reddy

The early and accurate diagnosis of plant disease is a crucial component of any crop management system. Plant diseases can be managed most effectively if control measures are introduced at an early stage of disease development. The detection of plant pathogenesis is by two ways: Based on their interaction with plants and methods based on their intrinsic property.

Detection methods based on intrinsic properties often have the advantages that they can easily be automated for application in large scale indexing. The most common methods are 1) Immunological assays and 2) molecular assays. Serological or immunological methods for detection of plant pathogens, particularly plant viruses have been available to plant pathologists for many years (Schaad 1979; Torrance & Jones 1981). However introduction of more advanced immunodiagnostic methods has expanded the scope of application to the diagnosis of diseases caused by many different viruses, bacteria, spiroplasma, phytoplasma and fungi (Clark 1981). There are some important fungal diseases, particularly those that are soil-borne and which affect the stem base, where diagnosis is difficult and early treatment is essential. Development of a quick diagnostic assays for such diseases would help to reduce both crop losses and unnecessary spraying. Immunological techniques are rapid, sensitive and specific, could be used for the rapid detection of fungal pathogens and diagnoses of the diseases, which they cause. They would also be helpful for research purposes, epidemiological studies, quantitative measurements and identification of non-fruiting mycelia which are common on plants roots.

The basic feature of the most commonly used and most widely applicable immunoassay are same as described in this chapter. The best known detection methods based on intrinsic properties are serological protocols using specific antibodies directed against antigenic sites on the coat protein of virus particles. The antibodies can, in general be raised from vertebrates that are injected with antigens. In most cases rabbits are used but when in the past large quantities were needed, sheeps, goats and even horses were used (Matt & Huttinga 1987). Chickens have been injected with antigens, and antibodies collected from the egg yolks (Bar-Joseph &

Malkinson 1980). Antibodies react specifically with the homologous antigen. Reaction however is not easy to detect. Therefore many protocols have been described to visualize the antibody antigen reaction

Antibodies are extraordinarily useful molecules. Their highly specific binding to a wide range of molecules, including peptides, carbohydrates, and nucleic acids, and their ability to identify specific antigenic determinants in these molecules make them extremely valuable.

Immunoassays utilizing polyclonal and/or monoclonal antibodies are being used extensively in plant virus diagnosis and have demonstrated tremendous utility in providing sensitive and specific analysis. Immunoassays have been improved and simplified over the past decade and now can be performed by persons with minimal training. The availability of monoclonal antibody technology and rapid, sensitive immuno assay formats has opened the door to development of sophisticated "high-tech" detection diagnostic tools.

Basic concepts of immunology and serology

Antigen: Foreign proteins or polysaccharides, which upon injection into an animal induce the production of antibody.

Antibody: Antibodies are modified serum globulins, which are produced in response to an antigen.

Antiserum: Blood serum containing antibodies is called antiserum.

Immunogenicity: The capacity of an antigen to induce an immune response.

Antigenicity: The property or the capacity to react specifically with an antibody.

Antigenic determinants or epitopes: Antigenic protein molecule will have several structural sites or reactivity residues which are known as epitopes.

Antigenic site: Specific amino acid sequences which specifically reacts to immunoglobulins.

Homologous antiserum; antiserum produced against a particular antigen (homologous).

Heterologous antiserum: Antiserum that reacts with other related antigens (heterologous) in addition to the specific antigen.

Antiserum titer: The titer of antiserum is the highest dilution of the antiserum that reacts with its antigen.

Immunoglobulins (Ig): The purified forms of antibodies are called

immunoglobulins. All immunoglobulins have a similar basic structure, consisting of two identical light (L) chain and two identical heavy (H) chains, which are linked together by non-covalent forces and disulfide bonds. Five classes of immunoglobulins (Ig) can be distinguished on the basis of five different types of heavy chain. These classes are IgG, IgA, IgM, IgD and IgE and their respective H chains are called $\gamma, \alpha, \mu, \delta$ and ϵ . The IgG and IgM immunoglobulins are the major types present in most sera and are the only ones that have so far been implicated in serological reactions with plant viruses.

Polyclonal antibodies (PACs)

Polyclonal antibodies are those obtained from serum of an animal following injection with an antigen, which contains many antigenic sites. PACs usually contain most of the immunoglobulin types and a large range of affinities for the different epitopes.

Advantages

1. Fairly easy and inexpensive to prepare,
2. They can be produced in a short time (3-6months),
3. They are useful in general diagnosis of related viruses or strains,
4. They heterogeneous with respect to antigen binding affinities and suitable for covalent attachment of enzymes, fluorochromes etc.

Monoclonal antibodies (MAbs)

A single antibody producing B-lymphocyte, immortalized either by mutation or fusion with a myeloma cell line, will produce antibodies (monoclonal) which react to a single epitope.

Production of monoclonal antibodies

1. Immunization of mice or rat
2. Culturing of mouse myeloma cell line
3. Extraction of spleen of immunized mouse
4. Fusion of spleen cells with myeloma cells
5. Fused cells (hybridoma) are grown in a selective medium that does not support the growth of non fused cells.
6. Supernatants of hybridoma cultures are screened for the presence of Mabs
7. Sub cloning of the hybridoma to ensure that the cell lines are monoclonal
8. Production of aseptic fluid for the use in detection.

Advantages of MAbs

1. Requirement for immunization-Mice or rats can be immunized with small amounts of antigen (100 μ g or partially purified virus).
2. Standardization and supply-MAbs provide a uniform reagent that can be distributed to different laboratories and can be produced in unlimited quantities.
3. Specificity -They combine only with one antigenic site so very specific to strains.
4. High affinity-The screening procedure for detecting MAbs permits selection of antibodies with very high affinity for the antigen
5. Storage - hybridomas can be stored in liquid nitrogen to provide a source of MAb producing cells over long period.

Disadvantages

1. Preparation-MAbs isolation is labour intensive, time consuming and relatively expensive.
2. Specificity -MAbs may be too specific for some applications, especially in diagnosis.
3. Sensitivity to conformational changes-They may be very sensitive to conformational changes in the antigen, brought about by binding to the solid phase or by other conditions in the assay.

Recombinant antibodies: Production of recombinant antibodies by linking DNA sequences which code for antibody binding regions onto generic immunoglobulin molecules and transferring the DNA into bacteria has introduced another set of advantages.

Phage displayed antibody: The molecular structures of antibodies have also made it possible to construct smaller antibody fragments, such as the Fab and single chain Fv(ScFv) which retain the antigen-binding properties of the IgG from which they were derived.

Fab and ScFv specific DNA sequence can be expressed in functional form in *E.coli*, allowing rapid protein engineering to reduce immunogenicity, increase affinity, or later specificity. ScFv has proven particularly useful, since they can be encoded in a single gene and yield a single polypeptide chain.

The V genes of MAbs can be used to construct recombinant Fab or ScFv antibody fragments while this approach has yielded many useful

antibodies for research. But the process is time consuming and inefficient. Only a relatively small number of antibodies are typically produced against a few dominant immunogenic epitopes. Use of MAbs to construct recombinant antibody fragments can be limited by difficulties in amplifying the heavy (V_H) and light (V_L) chain variable regions. Another is problems in cloning the V genes or low levels of expression in bacteria.

Phage display can also be used to make antibodies without prior immunization by displaying very large and diverse V-gene repertoires on phage and results in the ability to isolate antibodies with any desired specificity from a single phage display library.

Types of antigens and preparation of antigens

When plant pathologists become involved in immunology, the goal generally, is to generate an antibody probe, which will specifically identify a target antigen in an assay. This goal should be facilitated by an understanding of the basis of immunology and preparation of various antigens.

Considerations for selecting antigen: The selection of an antigen represents one of the very first steps in the preparation of antibodies. Antigens are critical to the process. At least four factors determine the effectiveness of particular component's antigenic characteristics. These are molecular size and structural relationships, foreignness, structural stability, and degradability. In addition, the nature of immune cell-cell interaction is a fundamental consideration for immunogenic activity.

Plant viruses: Plant viruses code for several proteins. Of these, the virion capsid and inclusion body proteins accumulate in the highest concentration in infected plants. The virion capsid proteins and whole virus are most frequently used as antigens. Inclusion body proteins have more potential for use as antigens than has some times been realized.

The main points in purification of these antigens are selection of source tissue, assays to monitor concentration and purity, procedures for purifying the virions or inclusion bodies, and when applicable, procedures for purifying the protein subunits of virion or inclusion bodies (Hiebert *et al.* 1984).

Cloning the gene and inserting it into an expression vector to produce the protein in *E. coli* or other suitable system have obtained other protein antigens encoded by the virus. Alternatively, a synthetic peptide

corresponding to portion of the virus protein whose structure has been deduced from the nucleic acid sequence may be used as the antigen (Ooshika *et al.* 1984). Availability of cloned cDNAs of plant virus genomes in principle allows production of any viral encoded proteins in expression vectors by standard methods of molecular biology (Sambrook *et al.* 1999). This approach has been used to produce antibodies to RNA replicase of brome mosaic virus. Another application involved production of antibody to a chimeric fusion protein of CMV protein 3A (Mac Kenzie & Tremaine, 1988).

Bacteria: Bacterial antigens may be extra cellular, intracellular, or structural and their biochemical nature may be protein, glycoprotein, polysaccharide, lipopolysaccharide or lipid. The usefulness of an antigen for serological detection depends on its properties and the serological test being used. For example, structural or somatic antigens normally work best for immunofluorescence and direct agglutination tests; soluble antigens are better in precipitin tests, ELISA and Immunodiffusion. Important properties of the antigen are: immunogenicity, location in the bacterium, copy number, solubility and stability.

Fungi: Probably the most significant reason for the lack of progress in development of fungal immunodiagnosics has been the difficulty of raising antisera that are species-specific. The common antigens used for antisera production are fungal spores, hyphal fragments, solubilized extracts of concentrate of culture filtrates and toxins. For specific, MAbs have been raised for laboratory based detection purposes, with specificity at the species level studies with *Pythium* and *Phytophthora* spp. indicate that it is easier to raise species specific MAbs to zoospore membrane than to cell walls.

Serological tests based on undefined antigens can be useful for diagnosis and detection work. However, purified antigens permit optimization of serological tests, accurate determination of specificity and precise delineation of strain relationships. The choice of test antigen depends on the objective of the test and the type of test being used. For identification of isolates and comparison of strains, pure cultures of bacteria are desirable. Concentration of antigen preparation should also be standardized. For serological diagnosis of bacterial plant diseases and detection of plant pathogenic bacteria in plant or soil samples, crude preparations can often be used directly without purification of antigen.

Production of antisera

Plant pathologists today can choose between polyclonal or monoclonal antibodies for serodiagnostic studies of plant pathogens. The choice of whether monoclonal or polyclonal antibodies are used must be based upon the type and purpose of assays.

Choice of animals: Any hemothermic animal can serve as a source of immune serum. Rabbits, chickens, guinea pigs, rats and mice are commonly used for production of immune serum. Rats, Mice and guinea pigs yield relatively small volume of serum. Chickens have high body temperature; serum requires high osmolarity for antigen-antibody reactions, which are detrimental to the integrity of some antigens. Larger volume of serum may be obtained from larger animals such as sheep, goats and horses but more antigens are required for immunization. Rabbits are used most often to produce immune serum because they can be housed and cared for with little effort.

Immunization: The amount of immunogen required depends on the characteristics of the antigen, the means by which it is prepared and the method used for injection. Longer immunization schedules requiring more antigens normally result in a greater variety and quantity of antibody than shorter schedules, but may produce antibody of lower specificity. A combination of injection methods is used to present immunogen to the antibody producing sites in the animals. Both intramuscularly and intravenous injections are usually combined in a series lasting 3-4 weeks. Intravenous injections result in the most rapid distribution of antigen to many sites, but antigen may be degraded quickly in the blood stream by the body defense systems. Therefore, more than 6-8 injections may be required to obtain serum of useable antibody concentrations. Procedures for immunizing rabbits with various plant pathogens are given in Table 1.

Table-1. Procedures for immunizing rabbits with plant pathogens.

Immunogen	Route number	Amount (mg/ml)
Viruses:		
Intact Virus particle		
Broad Bean wilt	1-IV, 4- IM*	46.2mg
Blueberry shoestring	3-IM	3.6mg
Kalanchoe latent	8-IP	2.0mg
Lettuce infectious yellows	4-IM	1.3mg
Maize dwarf mosaic	3-IM, 1-IV	4.0mg
Red Clover necrotic mosaic	2-IM, 2-IV	3.0mg
Sweet clover necrotic mosaic	3-IM, 2-IV	10.0mg
Turnip mosaic	4-IM	8.0mg
Capsid Proteins		
Alfalfa mosaic	3-IM	0.6mg
Maize stripe	3-IM	0.3mg
Papaya ringspot	2-IM	4.0mg
Non Capsid proteins		
Barley stripe mosaic	2-IM, 1-ID, 1-SC	0.6mg
Maize stripe- nucleoprotein	4-LL,	4.0mg
Maize stripe, rice hoja blanca	3-IM	1.0mg
Papaya ringspot, pepper mottle- amorphous inclusion	3(IM-TP)	3.0mg
Papaya ringspot, watermelon-1- cylindrical inclusion	3(IM-TP)	3.0mg
Sonchus yellow net-nucleocapsid gene	4-SC	4.0mg
Tobacco vein mottling - components	3-IM	2.0mg
Wheat streak mosaic- cylindrical inclusion	6-IM	3.1mg
Bacteria		
Bacterial Cells		
<i>Cornibacterium sepedonicum</i>	4-IM, 2-IV	3.0ml
<i>C. sepedonicum</i>	1-SC, 3-IV	4.5ml
<i>Erwinia carotovora pv. atroseptica</i>	3-IM, 2-IV	3.5ml
<i>Xanthomonas campestris pv. phaseoli</i>	6-IV	3.0ml
<i>X. campestris pv. Campestris</i>	4-IM	5.0ml
<i>X. campestris pv. Holcicola</i>	7-10 SC	3.5-5.0ml
Bacterial components		
X. c. pv. Campestris		
TCA extract	4 IP	7.5mg
Ribosomal extract	4 IP	12.0mg
Membrane protein	3 IP	1.2mg
PAGE-derived polypeptides	3 IP	0.7mg
<i>Clavibacterer nichiganese pv. insidiosum</i> extra cellular polysaccharide	1 IM, 2-IP	141-370 µg
Erwinia chrysanthemi		
Membrane protein	IM, IP	1.7mg
Rhizobium and Agrobacterium		
Ribosomal subunits	4-IM	12.0mg
Fungi		
<i>Ceratocystis ulmi</i> , toxin	3 IP	1.6mg
<i>Epichloe typhini</i> , mycelium	3 SC	3.0ml
<i>Eutypa armeniace</i> , cell wall	5 SC	22.0mg
<i>Gaeumannomyces graminis f. sp. tritici</i>	1 ID, 3 IM	2.1mg
<i>Ophiostoma ulmi</i> , toxin (Creatocystis)	2 IM	4.0mg
<i>Phaeoelis schweinitzii</i> , mycelium	2 IM	1.0mg
<i>Phomopsis longicola</i>	4SC	132.0mg

*IM= Intramuscular, IV = Intravenous, IP = Intraperitoneal, ID = Intradermal
SC = Subcutaneous, TP = Toe Pad, LN = Lymph Node

For intramuscular injection, the antigen preparation should be emulsified with an adjuvant (1:1). The most commonly used adjuvant is Freund's (incomplete) adjuvant which consists of paraffin oil and an emulsifier, mannide monooleate, complete adjuvant, in addition to these two components, contain heat killed *Mycobacterium tuberculosis*, or *M. butyricum* or a similar acid-fast bacterium. Emulsification with adjuvants results in very slow release of antigen, thereby stimulating excellent immune response. Antigen concentration required may vary from 500ug per ml. Nevertheless, for highly immunogenic viruses such as luteoviruses, even 100ig per ml will be adequate.

Blood collection and serum preparation: Blood is collected from rabbits, by making an incision in the marginal vein of the ear. It is preferable to collect the blood in sterile containers. The blood is allowed to clot at room temperature for 2 to 3 hours. After overnight refrigeration, the serum is collected with a Pasteur pipette and then centrifuged at 5000 rpm for 10 minutes.

Purification of immunoglobulins and antibody fragments: In some assays such as ELISA and Immunoelectron microscopy it is necessary to use purified immunoglobulins instead of whole antiserum. Some commonly used methods for purifying immunoglobulins are as follows:

1. **Ammonium sulfate precipitation:** Ammonium sulfate precipitation is the most commonly used procedure for preparing a crude immunoglobulin fraction from whole antiserum. By adjusting the salt concentration to $1/3$ or $1/2$ saturation, the globulins are precipitated, whereas the albumin and many other serum proteins will remain in solution.
2. **Rivanol precipitation:** Rivanol (2—ethoxy-6, 9-diaminoacridine lactate) is the soluble salt of an acridine base that is used for precipitating albumin and other serum proteins, while leaving the IgG in solution.
3. **Caprylic acid precipitation:** Under acidic conditions, the addition of caprylic acid to serum or ascetic fluid will precipitate most proteins, with the exception of the IgG molecules.
4. **DEAE cellulose chromatography:** After ammonium sulphate precipitation, antibodies are further purified by using the anion exchanger diethylaminoethyl (DEAE) cellulose. If the pH is kept at 6.5 only contaminants will bind to the DEAE cellulose. The separation of immunoglobulins can be effected by a batch procedure or on a column.

5. **Protein A chromatography:** Since protein A from the cell wall of *Staphylococcus aureus* has a strong affinity for antibodies, this property has been used for purifying immunoglobulins.

Storage of antisera: For long-term storage of antisera at 4°C, it is essential to add either glycerol (1:1) or sodium azide to a concentration of 0.02%. In lyophilized form, antisera can be stored at -20°C indefinitely. Antisera can be stored at -70°C for many years without losing potency. Antiserum mixed with an equal volume of glycerol can also be stored at -20°C. It is also advisable to store serum in small aliquots of 1.0 ml or less. Antisera should not be frozen and thawed repeatedly. This leads to aggregation of antibodies thereby affecting antibody activity by steric interference of the antigen combining site or by generating insoluble material which may sediment during centrifugation.

Labeling of immunoglobulins

Enzyme conjugates: The most commonly used enzymes are 1). Alkaline Phosphatase followed by 2). Horseradish Peroxidase. The presence of peroxidase and oxidizing substances in many plant extracts makes this enzyme less suitable in plant virology. The other enzymes, which are used, are 4). Penicillinase and a limited extent 4). Urease

Biotin conjugates: Since Biotin can be covalently linked to antibody without affecting its antigen binding capacity. Biotins labeled antibodies are superior reagents compared to enzyme labeled antibodies. The use of biotinylated viral antibodies increases the sensitivity of ELISA and over comes the narrow serotype specificity of DAS ELISA. Biotin, Avidin, Streptavidin are commonly used for labeling.

Europium conjugates: Antibody -Europium conjugates are used in time resolved fluoroimmuno assay to overcome the non-specific fluorescence originating from the sample.

Gold-labeled immunoglobulins: Gold labeled anti-virus monoclonal antibodies are used to localize by electron microscopy, for the exact positions of antigenic sites on virus particles

Types of immuno assays

The tests are based on the reaction between antiserum, a blood serum containing specific antibodies produced by injecting lab animals with a pure virus and an antigen-virus protein. The tests are specific since an antibody combines only with the antigen, which contains similar groupings of amino acid sequences. The main advantages of testing over bioassays

are that they provide rapid, accurate results, are less labour intensive and require few plant-growth facilities. The tests used for seed borne viruses can be grouped into different types: Flocculation tests in liquid media, immunodiffusion, labeled antibody techniques and immuno-electron microscopy (IEM).

Flocculation in liquid media: The combinations between antigen and specific antibody protein can be demonstrated in a variety of ways. One of the most direct methods is to observe the formation of a visible specific precipitate between the antigen and the antibody. Depending upon the size of interacting molecules two types of tests are recognized:

Precipitation is used to describe the insolubilization of macromolecules and virus particles; **Agglutination** refers to the clumping of cells or of particles of similar size.

Precipitin tests: In this tests the antigen is simply mixed with the antiserum at different dilutions and can be used to detect the viruses based on the formation of insoluble complex. The precipitin tests are: **tube precipitin tests, micro precipitin test, ring interface test or precipitin ring test:**

Agglutination tests: In these methods, materials such as bentonite, chloroplasts, RBC of sheep, gelatin, protein A or synthetic polystyrene latex are used as reaction sites. After the reaction takes place, the carrier particles exhibit agglutination or clumping. These tests are especially suitable for elongate virus particles, which do not diffuse through a gel medium. The agglutination tests include slide/chloroplast agglutination, latex agglutination, hemagglutination tests, passive hemagglutination tests and bentonite flocculation test:

Immuno diffusion in gels: Immuno diffusion tests are performed in semisolid media (gel). The reactants are allowed to diffuse and precipitin bands are formed wherever the reactants meet of suitable concentrations. These tests separate mixtures of antigen and antibodies by their size, diffusion coefficients and concentrations. These tests are more reliable and specific than the precipitin tests. Different types of immunodiffusions are *single diffusion, double diffusion, radial diffusion, immuno electrophoresis, immuno-osmophoresis and rocket immuno electrophoresis*

Labeled antibody techniques: Antibodies can be labeled to make virus-antibody aggregates readily observable, to obtain increased sensitivity or both. Antibodies labeled with fluorescent dyes, radioisotopes and enzyme labels have been used to detect seed borne viruses in individual seeds with accuracy and specificity. The various labeled antibody techniques are:

immuno fluorescent assays (IFA), radio immuno assays (RIA), enzyme immuno assays (EIA) and immuno binding assays (IBA).

Immuno fluorescent assays (IFA): Immuno fluorescent assays are widely used techniques for studying virus location and distribution within the tissues of host plants. They have now being employed for detecting and locating viruses in seed. These techniques are specific and provide detection by observing antigen-antibody reaction by chemically linking a fluorescent dye such as flourscein iso-thiocyanate (FITC) or Rhodamin B to specific antibodies. When the reaction is observed under fluorescent microscope, virus infection will show the fluorescence. This method has been applied for the detection of SqMV in embryos, cotyledons and seedlings of melons.

Radio immuno assays (RIA): Antibodies labeled with radioisotopes have been employed in detection of seed borne viruses. These techniques are very sensitive and are well suited to quantitative studies, but their application requires strict safety precautions and highly trained personncel, the conjugates have a short shelf life and expensive equipment is required to assist results.

Solid phase radio immuno assay (SPRIA): In this assay, the antibodies were used to coat polystyrene beads which were then submerged and incubated in crude seed extracts. If antigen was present, it will bind to the coating antibodies and could subsequently be detected by adding radioactive antibodies. The amount of radioactivity bound was directly proportional to the amount of virus present.

Radio immunosorbent assay (RISA): A simple and highly sensitive method has been described by Ghabrial and Shepherd (1980). It is a micro plate method based on the principle of DAS-ELISA and follows protocol of ELISA with the exeception that radio labeled antibody is substituted for the globulin enzyme conjugate. The test was critical for reliable detection of a single LMV infected lettuce seed in a group of 1000 seeds. This technique was also used for SqMV detection in cantaloupe seeds.

Enzyme Immunoassays

ELISA

Immuno binding assays (IBA): Blotting techniques have become widely used for the specific identification of proteins or viruses. These techniques have been simplified for routine detection of proteins/viruses by eliminating the electrophoresis fraction and transfer; the sample is directly applied to nitrocellulose membrane or filter paper as a spot, followed by detection through immunoprobe. The various IBA techniques are: Disperse dye immuno assay (DDIA), dot immuno binding assay (DIBA) and filter paper sero-assay (FiPASA).

Disperse dye immuno assay (DDIA): Disperse dye immuno assay differs from ELISA in the replacement of the enzyme by dye-sol particles and use of organic solvent instead of substrate incubation. The organic solvent dissolves the dye molecules from the dye-sol conjugate. DDIA has been used for detection of LMV in lettuce seed and PEBV in pea seed.

Dot immuno binding assay (DIBA): DIBA is another enzyme amplified serological detection system, which is widely used for virus detection. In this method, virus is directly bound to the nitrocellulose membrane and is detected with virus-specific antibodies that are in turn detected with virus specific antibody enzyme conjugates. The enzyme substrates used in DIBA form a spot of insoluble coloured product on the nitrocellulose membrane.

The use of nitrocellulose as the solid support for ELISA with a precipitating substrate found to be 2-4 times more sensitive than ELISA in micro titer plates. The virus is directly bound to the membrane and is detected with virus specific antibodies that are in turn detected with anti-species antibody enzyme conjugates. The enzyme substrates used in DIBA form a spot of insoluble coloured product on the nitrocellulose membrane.

DIBA is the simplest, no special equipment required and gives clear-cut positive or negative reaction and well suited to poorly equipped laboratories. DIBA has been applied successfully for the detection of BCMV in bean seed (Ligat & Randles, 1993). Ligat *et al.* (1991) developed DIBA for PSbMV detection with a detection limit of about 32ng per 1ul sample.

Filter paper sero-assay (FIPASA): Plant viral antigens are readily detected on filter or chromatography paper with a simple procedure adapted from a method developed for serological comparisons of insoluble proteins disks, the paper air-dried, then incubated with antiserum. After washing to remove excess antibody, the paper is incubated with protein A-peroxidase, washed, and incubated with 4-chloro-1-naphthol substrate. Upon development, violet blue spots centered on the point of original application of presumed antigen, identify those antibody specific reactions. This technique can be used to detect viral antigen in plant sap, seed embryos and preparation of varying degrees of purity. The method is inexpensive and rapid requiring as little as 2 hrs. The method has been applied for detection of barley stripe mosaic virus in barley embryos and SbMV.

Immuno electron microscopy (IEM): The visualization of immunological reactions on electron microscope grids is one of the most sensitive serological techniques. Two different approaches can be distinguished, depending on whether the viral antigen is in suspension or is visualized in thin sections or

infected tissue. There are three general methods viz. clumping, antibody coating or decoration and trapping or ISEM, which are used for virus detection in seed.

Clumping of virus particles or Antiserum-virus mixtures: Formation of virus- antibody complexes, when a virus preparation is mixed with a suitable dilution of specific antiserum, can be visualized in the electron microscope by the appearance of clumps of varying sizes.

Decoration of antibody coating: The method involves confirming the identity of virus particles by coating with antibodies after preparation of specimen grids. The prepared grids are floated on drops of suitably diluted antiserum. Antibodies stick to the surface of the virus particle and, after negative staining, can be seen coating them.

Immunosorbent electron microscopy (ISEM) or trapping: The trapping of plant viruses to electron microscope grids coated with specific antiserum was first described by Derrick (1973), which was later renamed as ISEM (Roberts *et al.* 1982). In this procedure, support films are precoated with specific antibody to which homologous virus particles in the extract become attached and concentrated. Such successive fixing of antibody and antigen allows interpolation of washing stages to remove salts or other undesirable components.

IEM is a rapid, reliable and sensitive method for testing of seed for virus infections. The crude antiserum can be used directly without fractionation. Even antiserum with relatively low titers can be used successfully and the presence of a certain level of nonspecific antibodies may not interfere with the result.

Selected references

- Hampton R, Ball & De Boer S 1990 In: *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens*. Laboratory Manual, APS Press, USA. 389 pp.
- Huttinga H 1996 *Advances in Plant Pathology* 23: 59-71.
- Miller S A & Martin R R 1998 *Ann. Rev. Phytopath.* 26: 400-432.
- Duncan J M & Torrance L 1992 In: *Techniques for the Rapid Detection of Plant Pathogens*. Blackwell Scientific Publications, Oxford, U.K.

PCR based detection of plant viruses

A Ishwara Bhat

A virus is a nucleoprotein that is too small to be seen with a light microscope, multiplies only in living cells and has the ability to cause disease. Viruses do not divide or do not produce any kind of specialized reproductive structures such as spores, but they multiply by inducing host cells to form more virus particles. About 1000 different viruses are known to infect various plant species. Viruses are either elongate, spherical or bacilliform in shape and consist of nucleic acid (either RNA or DNA) and a protein, and a few of them contain additional chemical compounds such as polyamines, lipids or enzymes. Viruses are transmitted from plant to plant in a number of ways such as vegetative propagation, mechanically through sap, and by seed, pollen, insects, mites, nematodes, dodder and fungi.

Plant viruses differ greatly from all other plant pathogens in size, shape, composition, physical structure, methods of infection, multiplication, translocation within the host, dissemination and symptoms they produce on the host.

Need for diagnostics for virus detection

Correct diagnosis is a pre-requisite for effective management of any disease. The causal organism involved in fungal and bacterial diseases can be easily identified with a light microscope. But this is not the case with virus as they cannot be seen under light microscope. Besides, in most cases symptoms caused by viruses resemble those caused by mutations, nutrient deficiencies or toxicities. As viruses are obligate parasites and always systemic in nature, once infected virus particles will be present throughout the plant system. Unlike fungi and bacteria, a plant infected with a virus can not be cured because of lack any effective viricide. Hence planting of virus-free healthy material is very important especially in cases when virus gets transmitted from one generation to another through the use of infected vegetative propagating material or seed. In order to check the virus-free nature of the planting material, use of quick, sensitive and reliable diagnostic methods are essential.

Diagnostic methods

The methods for detecting plant viruses involve primarily the transmission of the virus from diseased to healthy plant by grafting, or by rubbing with plant sap or transmission through dodder or insect vectors. However, the most definitive proof of the presence of virus in a plant is provided by its purification, electron microscopy, serology, nucleic acid hybridization and polymerase chain reaction. Choice of planting material to be sampled is very important for successful detection and diagnosis. Sensitivity (how small an amount of virus can be detected), accuracy, reproducibility, number of samples that can be processed in a given time, adaptability to field conditions, cost and degree of operator training required are some of the factors one need to consider while selecting a method for detection. Among the methods, PCR based methods are the most sensitive.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is the most sensitive method (10^2 - 10^5 times more than ELISA) available presently for the detection. It is important when viruses occur at low concentration. It also has the potential to detect more than one virus in one reaction and diagnosis is amenable to automation. PCR technique involves the specific amplification of the target DNA fragment enzymatically under *in vitro* conditions. The method involves the hybridization of synthetic complementary oligonucleotide primers to the target sequence and synthesis of multiple copies of cDNA of the sequence between primers using heat stable DNA polymerase. Each amplification cycle consists of melting the double stranded template DNA at high temperature, annealing (hybridization) of the primers with complementary sequences in the template DNA at low temperature, extension of the primers with DNA polymerase (DNA synthesis). During each cycle, the sequence between the primers is doubled. A normal PCR involves 30-50 such amplification cycles.

PCR for detection of

Plant viruses containing single or double stranded DNA as their genomes in the genera such as *Badnavirus*, *Begomovirus*, *Caulimovirus*, *Curtovirus*, *Mastrovirus*, *Nanoviruses*, can be used as template for amplifying either full or partial genomes of these viruses. As sequences of many species of viruses infecting different crops of above virus genera are already available, primers can be designed to any region of genome for their amplification through PCR. The important steps in the PCR for plant viruses containing either single- or double stranded DNA as their genomes include:

(i) extraction of total DNA from virus infected plant or from purified virus preparations; (ii) synthesis of two virus specific primers; (iii) set up the PCR reaction in a vial by adding extracted nucleic acid, primers, nucleotides, magnesium chloride and *Taq* polymerase. Use nucleic acid extracted from a known infected and healthy plants to serve as positive and negative controls respectively; (iii) keep the vials in the PCR machine and start the run as per the pre-decided program and (iv) identify positive reactions by running contents of PCR on the agarose gel. The presence of bands at the expected position indicate that sample under test is positive.

Reverse transcription polymerase chain reaction (RT-PCR) for detection of RNA viruses

RT-PCR is a method to amplify complementary DNA (cDNA) copies of RNA. In this, the first step is the enzymatic conversion of RNA to a single stranded cDNA template. An oligo deoxynucleotide primer is hybridized to the mRNA and is then extended by an RNA-dependent DNA polymerase (Reverse transcriptase) to create cDNA copy that can be amplified by PCR. Either RNA isolated from purified viral preparations or total RNA isolated from infected plant or dsRNA isolated from infected plant can be used as template to initiate cDNA synthesis. The primer for first strand cDNA synthesis can be specifically designed to hybridize to a particular target gene or it can be an oligo (dT) (for viruses containing Poly (A) tail at their 3' ends). Amplification of the desired portion of cDNA can be achieved in PCRs primed by sense and antisense oligonucleotide primers corresponding to specific sequences in particular cDNAs. For maximum specificity, the antisense primer should be located upstream of the oligonucleotide used to prime cDNA synthesis. Positive and negative controls should always be included when setting up RT-PCRs.

Immunocapture (IC) PCR or IC-RT-PCR for detection of viruses

In this method, prior to PCR, virus particles are captured, immobilized and concentrated on a sterile solid surface previously coated with virus specific antiserum. Procedure has been successfully used for the amplification of several plant viruses. Thin walled tubes or microtitre plates are coated with 100 μ l of virus specific antiserum and incubated overnight at 4°C. The tubes are then washed and added with extracted plant sap from infected plant and incubated for 3-4 h at room temperature. Samples are removed and tubes are washed before adding the required quantity of water. To release viral nucleic acid, 2-3 cycles of freezing (-80°C for 10 min) and thawing (70°C for 5 min) are given. Alternatively, viral nucleic acid can be released by addition of Tris-HCl and 1% Triton X-100 and by incubation at

65°C for 10 min with vortexing. This is followed by either PCR or RT-PCR. Recently, real time quantitative PCR and multiplex PCR is becoming more popular for detection of viruses. One can choose the best method, depending on the sensitivity required, number of samples to be tested, types of sample, reliability of the technique, equipment, cost and expertise available.

Selected references

- Atzmom G, van Oss H & Czosnek H 1998 *European J. Plant Pathol.* 104: 189-194.
- Bhat A I, Jain R K & Ramiah M 2002 *Indian Phytopath.* 55: 216-218.
- Hu J S, Wang M, Sether D, Xie W & Leonhardt K W 1996 *Ann. Appl. Biol.* 128: 55-64.
- Jain R K, Pappu S S, Pappu H R, Culbreath A K & Todd J W 1998 *Plant Dis.* 82: 900-904.
- Mumford R A, Barker I & Wood K R 1996 *J. Virol. Methods* 57: 109-115.
- Nalasco G, de Blas C, Torres V & Ponz F 1999 *J. Virol. Methods* 45: 201-218.
- Rowhani A, Maningas M A, Lile L S, Daubert S D & Golino D A 1995 *Phytopathology* 85: 347-352.
- Sharman M, Thomas J E & Dietzgen R G 2000 *J. Virol. Methods* 89: 75-88.
- Singh R P, Kurz J, Boiteau G & Moore L M 1997 *American Potato Journal* 74: 305-313.

ITS based species identification of *Radopholus similis*

Santhosh J Eapen

The burrowing nematode (*Radopholus similis*), an obligate migratory endoparasite, is widely prevalent in most of the tropical and sub tropical regions of the world. Accurate and reliable identification of the pest is fundamental to many aspects of the nematode management. DNA based identification methods are very sensitive, being able to detect a single nematode egg or second stage juvenile.

Principle

For molecular analysis it is useful to study specific genomic regions. One such region is the ribosomal DNA (rDNA) gene family. The rDNA gene family is a multigene family consisting of many copies of genes encoding for three ribosomal components 28S, 5.8S and 18S (Files & Hirsh 1981). In most eukaryotes, the 5' to 3' organization of the gene family is an external transcribed spacer (ETS); the 18S gene; an internally transcribed spacer (ITS1); the 5.8S gene; ITS2; 28S gene; and the intergenic spacer (IGS). The IGS region is followed by another copy of the gene family.

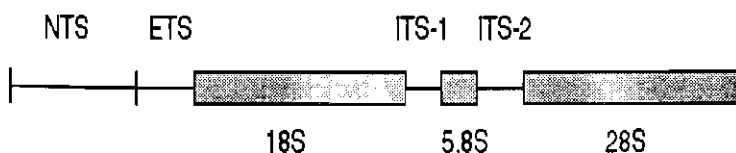


Fig. 1. Diagram of the ribosomal DNA gene family. The regions coding for the 5.8S, 18S, and 28S subunits of rRNA are shown by bars; NTS = non-transcribed spacer, ETS = external transcribed spacer, ITS = internal transcribed spacer regions.

Highly conserved regions in the ribosomal repeat array can be used for study of relationships across phyla more variable regions can be used at lower taxonomic levels. The ITS region does not encode for any product, permitting it to evolve at a faster rate than the ribosomal coding regions.

The level of variation in this region makes it suitable for detecting genetic variation among genera, species and within species.

Materials required

Nematode culture: Burrowing nematodes, *R. similis*, were collected from infected black pepper (*Piper nigrum* L.) roots and were cultured on excised carrot disk. The nematodes were extracted from carrot disks after multiplication and were used for DNA isolation.

Equipments: Water bath, Microfuge, Electrophoresis unit, Biophotometer, Thermal cycler, Pipettes, micro centrifuge tubes, sterile tips, micro homogeniser

Extraction Buffer

- 100mM Tris-Cl pH 8
- 100mM EDTA
- 100mM NaCl
- 0.5% SDS
- 200 µg of proteinase K

Buffer saturated phenol, Chloroform:Isoamyl alcohol (24:1), Isopropanol, 70% alcohol, Sterile ultrapure water, Taq buffer (10X), B.S.A (10 mg/ml), dNTP mix (2.5mM), Forward primer and reverse primer (10 pm/µl), Taq DNA polymerase (3 u/µl), Template DNA, PCR tubes.

Details of protocol

PCR amplification

1. PCR is performed in a 10 µl reaction using the recipe given in Table 1 and temperature profile as given in Table 2.
2. Resolve the product on a 1% Tris Acetate EDTA buffered agarose gel.

Table 1. Recipe for amplification r DNA region of *R.similis*

Reagents	Stock Concentration	Required Concentration	Required Volume
Sterile ultra pure water	1 X	1 X	5.23 μ l
Taq buffer with 15mM MgCl ₂	10 X	1 X	1 μ l
BSA	1mg/ml	40 μ g	0.4 μ l
dNTP mix	2.5 mM	200 μ m	0.8 μ l
Forward primer	10 pm/ μ l	2 pm	0.2 μ l
Reverse primer	10 pm/ μ l	2 pm	0.2 μ l
Taq DNA polymerase	3 U/ μ l	0.5 U	0.17 μ l
Template DNA	50ng/ μ l	100ng	2 μ l

Table 2. Temperature profile for amplification of rDNA region of *R. similis*.

	Temperature	Time
Initial denaturation	94°C	2 minutes
Followed by 35 cycles of		
Denaturation	94°C	30 seconds
Annealing	65°C	1 minute
Extension	72°C	1 minute
And final extension of 72°C		10 minutes

Anticipated result and interpretation of data

The forward primer binds to ITS 1 region and the reverse primer to 5.8S region of ribosomal gene of *R. similis*. The primer set amplifies partially ITS 1 and 5.8 S region of ribosomal gene of *R. similis*. A product of 398 bp will be obtained when *R. similis* genomic DNA is amplified using the specific primers.

Selected references

- Files J G & Hirsh D 1981 *J Mol Biol.* 149: 223-240.
Pastrik K H, Rumbenhorst H J & Burgermeister W 1995 *Fund. Appl. Nematol.* 18: 109-114.

Applications of bioinformatics in diagnostics

Santhosh J Eapen

Conventional biochemical methods and phenotypic tests for species differentiation are tedious and time-consuming and may require specialized testing that is beyond the capacity of many. Recent progress in molecular biology and bioinformatics allows the consideration of other methods that are more universal and less time-consuming.

Molecular methods such as PCR based methods, restriction pattern analysis etc. are widely employed today for taxonomic purposes. *In silico* approaches are adopted on a routine basis in activities like design of primers, drawing dendrograms etc. that are essential in most molecular methods (Please see separate chapters on these).

Of late, the advances in DNA sequencing technology have greatly enhanced the ability to determine the identity of several organisms (Kolbert *et al.* 1999). The availability of sequence data from related genomes has facilitated comparative genomic analysis, which not only allows the study of major evolutionary processes, but also the determination of proteins conserved across - or unique to different species. Today, sequencing techniques are well developed, but the identification tasks require the chaining of different programs that are sometimes complex to handle, and hence bioinformatics tools are highly relevant.

a. Sequence databases: For accurate gene sequence identification of organisms, we are dependent on accurate sequences in databases, appropriate names associated with those sequences, and an accurate sequence for the isolate to be identified. There are several reasons for errors in unverified databases such as GenBank, which accept any linked name and sequence that is sent to them. Therefore, it is suggested to develop and use peer-reviewed databases for that would improve the validity of this method of organism identification. A detailed list of molecular biology databases is published every year in the journal - *Nucleic Acids Research* (Galperin 2006). Some of the customized *in silico* tools and databases pertaining to plant pathogens and biocontrol agents are listed in Table 1.

b. *Sequence based methods*: These are methods wherein a lot of sequence analyses to be performed by drawing data from databases like GenBank. Bioinformatics tools automate and speed up different operations for the treatment of sequences. **RIDOM** (Ribosomal Differentiation of Medical Microorganisms) is an evolving electronic resource designed to provide microorganism differentiation services for medical identification needs using ribosomal DNA sequence (Harmsen *et al.* 2002). Resulting from a similarity search, a species or genus name for the specimen in question will be returned. **BIBI** (Bioinformatics Bacterial Identification) is one such tool that combines similarity search tools in the sequence databases and phylogeny display programs (Devulder *et al.* 2003). In addition, to automate the sequence analysis, BIBI integrates different sequence databases which are specifically adapted to bacterial identification to eliminate inaccuracies related to the direct use of sequences from GenBank.

HoSeqI (<http://pbil.univ-lyon1.fr/software/HoSeqI/>) is a web service allowing to automatically assigning sequences to homologous gene families from a set of databases (Arigon *et al.* 2006). After identification of the most similar gene family to the query sequence, this sequence is added to the whole alignment and the phylogenetic tree of the family is rebuilt. Thus, the phylogenetic position of the query sequence in its gene family can be easily identified.

Table 1. List of some databases and tools pertaining to taxonomy and identification of major plant pathogens and biocontrol agents.

Bacteria	
ICB	http://seasquirt.mbio.co.jp/icb/index.php
MICdb	http://210.212.212.7/MIC/index.html
BSORF	http://bacillus.genome.jp/
SubtList	http://genolist.pasteur.fr/SubtList/
<i>Bacillus cereus</i> MLST database	http://pubmlst.org/bcereus/
<i>Pseudomonas</i> Genome database	http://v2.pseudomonas.com/search.jsp
PseudoCAP	http://www.pseudomonas.com/
BIBI	http://pbil.univ-lyon1.fr/bibv/
<i>BacMap</i>	http://wishart.biology.ualberta.ca/BacMap/index.html
<i>Comprehensive Microbial Resources</i>	http://cmr.tigr.org/tigr-scripts/CMR/CMRHomePage.cgi
<i>Integrated Microbial Genomes</i>	http://img.jgi.doe.gov/cgi-bin/pub/main.cgi
<i>Microbial Genome Projects at the BCM</i>	http://www.hgsc.bcm.tmc.edu/projects/microbial/
HGSC	
Viruses	
DPV web	http://www.dpvweb.net/
Plant virus biodiversity & ecology	http://www.genome.ou.edu/pvbe.html
NCBI Viral Genomes	http://www.ncbi.nlm.nih.gov/genomes/VIRUSES/viruses.html
VIDA	http://www.biochem.ucl.ac.uk/bsn/virus_database/VIDA.html
VIPERdb	http://viperdbscripps.edu/
VirGen	http://bioinfo.ernet.in/virgen/virgen.html
<i>Plant Viruses Online</i>	http://image.fs.uidaho.edu/videl/
<i>Retroviruses</i>	http://www.ncbi.nlm.nih.gov/retroviruses/

Oomycetes	
The Phytophthora Genome Consortium Database	http://www.pfgd.org/pfgd/
<i>Phytophthora</i> database	http://www.phytophthoradb.org/
PhytlD	http://www.phytlid.org/list.asp
<i>Phytophthora</i> DB	http://phytophthora.vbi.vt.edu/
Phytophthora Functional Genomics Database	http://www.pfgd.org/
VMD	http://phytophthora.vbi.vt.edu/
OGD - Oomycete Genomics Database	http://www.oomycete.net/
Fungi	
TrickOKEY	http://www.isth.info/tools/molkey/index.php?do=unset
TrichoMARK	http://www.isth.info/tools/blast/index.php
<i>Trichoderma reesei</i> EST database	http://trichoderma.iq.usp.br/
Mycorrhizal database	http://www.sou.edu/BIOLOGY/Faculty/Southworth/mycorridata.htm
CADRE- Central Aspergillus Data Repository	http://www.cadre.man.ac.uk/
COGEME Phytopathogenic Fungi and Oomycete EST Database	http://cogeme.ex.ac.uk/
FGDB - <i>Fusarium graminearum</i> Genome Database	http://mips.gsf.de/genue/proj/fusarium/
PHI-base	http://www4. Rothamsted.bbsrc.ac.uk/phi-base/
Fungal Plant Pathogen Database	http://fppd.cbio.psu.edu/
Fungal Genome Initiative	http://www.broad.harvard.edu/annotation/fgi/
Nematodes	
PartiGene	http://www.nematodes.org/NeglectedGenomes/NEMATODA/index.shtml
NemBase	http://www.nematodes.org/nematodeESTs/nembase.html
Nematode.Net	http://www.nematode.net/index.php
WormBase	http://www.wormbase.org/
PPNEMA - Plant-Parasitic Nematode rRNAs	http://highost.area.ba.cnr.it/PPNEMA
NemaTOL	http://nematol.unh.edu/
General	
NCBI Taxonomy Browser	http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html
Ribosomal Database Project (RDP-II)	http://rdp.cme.msu.edu/
RIDOM	http://rdp.cme.msu.edu/
RISSC	http://ulises.unh.es/RISSC
Tree of Life	http://phylogeny.arizona.edu/tree/phylogeny.html
PathPort, pathogen portal project	http://pathport.vbi.vt.edu/main/home1.php
PHI-base. Pathogen Host Interaction	http://www.phi-base.org/
Plant Pathogen Genomics	http://cpgr.tigr.org/project_summary.html
Plant Pathogens (TIGR)	http://www.tigr.org/~vinita/PPwebpage.html

DNA-BAR (<http://dna.engr.uconn.edu/~software/DNA-BAR/>) is a software package for selecting DNA probes (or distinguishers) that can be used in genomic-based identification of microorganisms (Dasgupta *et al.* 2005). Given the genomic sequences of the microorganisms, DNA-BAR finds a near-minimum number of distinguishers yielding a distinct hybridization pattern for each microorganism. Selected distinguishers satisfy user specified bounds on length, melting temperature and GC content, as well as redundancy and cross-hybridization constraints.

Single nucleotide polymorphisms (SNPs) are the most abundant form of genetic variations in closely related species, strains or isolates. Some SNPs confer selective advantages for microbial pathogens during infection and many others are powerful genetic markers for distinguishing

closely related strains or isolates that could not be distinguished otherwise (Song *et al.* 2005). **SNPsFinder** (<http://snpsfinder.lanl.gov/>) is a web-based application for genome-wide identification of SNPs. It can generate PCR primers for all predicted SNP regions according to user's input parameters to facilitate experimental validation.

The **ORFanage** database (<http://www.cs.bgu.ac.il/~nomsiew/ORFans>) includes about 32,000 unique ORFs (ORFans) from 84 fully sequenced microbial genomes (Siew *et al.* 2004).

- c. *Proteomics based methods*: Mass spectrometry data are combined with information from genome and proteome databases in a new rapid method for identification and characterization of viruses, spores, vegetative bacteria and fungi (Demirev *et al.* 1999). In another approach MALDI-TOF mass spectrometry has been coupled with Internet-based proteome database search algorithms accounting for post-translational modifications in putative protein biomarkers were successfully used for direct identification of *H. pylori*, a human pathogen (Demirev *et al.* 2001). Pattern recognition tools in bioinformatics have been applied for species specific bacteria identification using differential mobility spectrometry (Shnayderman *et al.* 2005). The pattern discovery/recognition algorithms (**ProteomeQuest**) are used to analyze headspace gas spectra generated by micro-machined differential mobility spectrometer to reliably discern multiple species of bacteria in vitro. The **Procom** (<http://ural.wustl.edu/~billy/Procom/>) database compares proteins from thirty completely sequenced eukaryotic genomes and identifies unique species- or clade-specific proteins (Li *et al.* 2005). **CUPID** (**C**ore and **U**nique **P**rotein **I**dentification) (<http://pir.georgetown.edu/cupid>) is another computational tool that provides sets of proteins unique to a strain, species, and genus level (Mazumdar *et al.* 2005).

Selected references

- Arigon A M, Perriere G, Gouy M 2006 *Bioinformatics* 22 : 1786-1787.
DasGupta B, Konwar K M, Mandoiu I I, Shvartsman A A 2005
Bioinformatics 21 : 3424-3426.
Demirev P A, Demirev Y P, Ho V, Ryzhov C, Fenselau Y P 1999 *Analytical Chemistry* 71 : 2732-2738.

- Demirev P A, Pineda F J, Lin J S, Fenselau C 2001 *Analytical Chemistry* 73 : 4566-4573.
- Devulder G, Perrière G, Baty F, Flandrois J P 2003 *J. Clinical Microbiology* 41 : 1785-1787.
- Galperin M Y 2006 *Nucleic Acids Res.* 34 : Database issue D3–D5.
- Harmsen D, Rothgänger J, Frosch M, Albert J 2002 *Nucleic Acids Res.* 30 : 416-417.
- Kolbert C P, Persing D H 1999 *Curr. Opin. Microbiol.* 2 : 299-305.
- Li J B, Zhang M, Dutcher S K, Stormo G D 2005 *Bioinformatics* 21 : 1693-1694.
- Mazumder R, Natale D A, Murthy S, Thiagarajan R, Wu C H 2005 *BMC Bioinformatics* 6 : 279.
- Siew N, Azaria Y, Fischer D 2004 *Nucleic Acids Research* 32 Database issue: D281-283.
- Shnayderman M, Mansfield B, Yip P, Clark H A, Krebs M D, Cohen S J, Zeskind J E, Ryan E T, Dorkin H L, Callahan M V, Stair T O, Gelfand J A, Gill C J, Hitt B, Davis C E 2005 *Analytical Chemistry* 77 : 5930-7.
- Song J, Xu Y, White S, Miller K W P, Wolinsky M 2005 *Bioinformatics* 21 : 083-2084.

IT based species identification

Santhosh J Eapen

Species identification lies at the heart of most ecological and biological studies, but it is recognized as a difficult and often frustrating task. The routine identification of specimens of previously described species has many of the characteristics of other activities that have been automated, and poses a major constraint on studies in many areas of both pure and applied biology. Taxonomists have sought to overcome the difficulties of species identification by developing a range of tools and techniques that have increasingly involved the use of computers. Where possible, automation has been a common response of humankind to many activities that have to be repeated numerous times (Gaston & O'Neill 2004). Although automated species identification is a realistic option, there are some very real technical obstacles yet to be overcome.

Role of IT

The progress in the development of automated species identification is extremely encouraging that such an approach has the potential to make a valuable contribution to reducing the burden of routine identifications. IT can be applied in diagnostics with varying objectives (Scott 1998).

- a. Handling facts to produce information like in the case of nomenclature databases
- b. Interpreting information to produce knowledge for example taxonomic information systems
- c. Using knowledge to support decision making as in diagnostic tools
- d. Using knowledge to make predictions as in the case of risk analysis
- e. Passing on knowledge in education and training using web, e-publishing etc.

IT tools in diagnostics

Botanists started applying computer tools for taxonomy and identification first. An important milestone in the history of identification by computer was the development of DELTA (The Description Language

for Taxonomy) (<http://delta-intkey.com/> or <ftp://delta-intkey.com/>) format by Dallwitz in 1980. This was later accepted as the international data standard for identification data.

Different types of identification methods are in vogue but most of them fall into one of the two broad groups, comparison or step-by-step elimination (Pankhurst, 1998). The comparison method is used when an unknown specimen is compared with a voucher specimen or illustrations. The matching method is possibly the most accurate of all methods, since it makes use of all the available information. Identification methods using probability are also matching methods. The familiar dichotomous key is an example of elimination method but is error prone. The conventional identification keys use a tree with characters at the internal nodes and taxon names at the terminal nodes.

Online diagnostic keys

These are electronic versions of conventional dichotomous keys. The leads of the existing key are displayed in turn as characters are typed in. Other than the convenience they are not preferred as all the disadvantages of diagnostic keys are preserved.

Multi-access keys

These are the most popular type and are interactive and online. Multi-access keys do not suffer from the unanswerable couplet problem encountered in dichotomous keys because they allow you to start with any character you choose and to proceed in any order you choose. Thus, in using a multi-access key you can avoid characters that are difficult for you or not appropriate for your specimen, making a multi-access key superior to a dichotomous key. Ultimately, a multi-access key eliminates all taxa except the one that matches your specimen. However, if you cannot eliminate all the taxa, you will be left with a small group of taxa that can be compared more closely. Since multi-access keys depend on a complete check of all the taxa in the key's database each time a question is answered, they are ideal for computers, which can easily handle repetitive tasks at high speed.

Hypertext keys

Hypertext keys are multi-access keys wherein the keywords are hyperlinked to additional information like descriptions, illustrations or images. Any multimedia can be linked like this for improved comparison and accuracy.

Expert systems

An expert system is a program that uses available information, heuristics, and inference to suggest solutions to problems in a particular

discipline. They are now extensively used in species identification. Of the three main types of expert system available, the frame-based system is the most appropriate model for a taxonomic expert system rather than a logic- or rule-based system. A rule-based system requires the original knowledge (species descriptions) to be structured into rules, whereas a frame-based system can store the generic and specific descriptions in a series of frames. The frames fall into a hierarchy which closely resembles the taxonomic hierarchy, and down which information can be inherited. Two aspects of frame-based systems considered are the use of probabilities in identification, and the optimum structure of the knowledge base. The conventional use of probabilities is to provide an indication of the correctness of the result. However, in some studies involving the identification of many specimens, the speed of identification may be increased (with a reduction in accuracy) if identifications are made to a predetermined probability level. Although frames allow accurate representation of the taxonomic hierarchy, a semantic net, incorporating structures of the organism and/or details of the habitat may result in a more efficient expert system.

Neural networks

A neural network real or virtual device, modeled after the human brain, in which several interconnected elements process information simultaneously, adapting and learning from past patterns. An Internet-accessible automated identification system that uses neural networks to make species identifications based on digital images is under development at the American Museum of Natural History. The goal is to create a system that can identify species without requiring the user to have more than the most basic knowledge of the organisms to be identified. CAPSI (Computer - Aided Plant Species Identification) (CAPSI) is an intelligent system developed recently for identifying plants based on leaf shape matching technique.

Some tools

Lucid 3 is a matrix key, the process of making identification involves selecting those states from a list of character states that best describe the specimen to be identified.

Availability: Centre for Biological Information Technology, The University of Queensland, Queensland, AUSTRALIA

Lucid Phoenix (Dichotomous) key system is also Java-based and is accessed via an applet player. However, while new dichotomous keys can be built using this tool, the main reason for developing *Lucid Phoenix* has been to take advantage of the thousands of dichotomous keys that already exist. Published dichotomous keys can be converted into interactive, web-based

or CD-based keys by first scanning the published key, using optical character recognition (OCR) software to convert it into a text file, and then importing it into the builder. A single key stroke will then deploy the key in the applet player, producing a key which is interactive, can be enhanced with multimedia, and delivered seamlessly across the Internet.

Availability: Centre for Biological Information Technology, The University of Queensland, Queensland, AUSTRALIA

Electronic Field Guide (EFG), developed at the University of Massachusetts, Boston, is a tool that allows researchers to build their own online keys from observations, measurements, images, and publications.

Availability: <http://efg.cs.umb.edu/index.html>

Linnaeus II is an innovative multifunctional research tool for taxonomists and biodiversity researchers. It facilitates biodiversity documentation and species identification.

Availability: Expert centre for Taxonomic Identification (ETI), Amsterdam, The Netherlands (<http://www.eti.uva.nl/products/linnaeus.php>).

Some of the important IT resources pertaining to diagnostics are listed in Table 1.

The IISR initiatives

The Bioinformatics Centre at IISR, Calicut has taken some initiatives in this direction recently. *Phytfinder* (<http://www.spices.res.in/pi-/phytfinder.htm>) is a multi-access, interactive, online tool for identification of *Phytophthora* species. PhyDisH (<http://www.spices.res.in/phydish/>) is an online database of *Phytophthora* diseases of horticultural crops. The offline database on *Piper* spp., PiperBase, contains a hypertext key for identification of *Piper* species of South India. Work is in progress for developing an expert system for identification of rhizobacteria.

Conclusion

The Web-based tools allow users with minimal training and technical knowledge to identify, report, and map species information. Using these tools anybody can correctly identify species by matching the sample they've collected to the descriptions and photos provided. Internet-accessible automated identification systems have the potential to facilitate studies of diverse taxa and to lead to an explosion of knowledge about our biodiversity.

The full potential of taxonomy will be realized when institutions supporting taxonomic research and biological collections are components of one comprehensive network. Nodes of the network will be existing

institutions with biological collections and taxonomic research programs. Each node will have unique strengths and expertise, e.g., particular taxa, geographic data, or specialized instrumentation. The network will be distributed across the nation, and resources at each node (e.g., specimens, images, literature, molecular data) will be available to researchers, educators, and policy-makers everywhere via the Internet.

Table 1. Some IT resources pertaining to taxonomy and identification of plant pathogens and biocontrol organisms.

Name of the resource	URL
Bacterial Nomenclature up-to-date	http://www.dsmz.de/microorganisms/bacterial_nomenclature.php
World <i>Phytophthora</i> Genetic Resource Collection Database	http://phytophthora.ucr.edu/databasemain.html
ICTVdB	http://www.ncbi.nlm.nih.gov/ICTVdb/Wikikey/index0.htm
Virology on www	http://www.virology.net/garryfavwebplant.html
Plant viruses online	http://image.fs.uidaho.edu/videl/
<i>Trichoderma</i>	http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm
Mycorrhizal database	http://www.sou.edu/BIOLOGY/Faculty/Southworth/mycordata.htm
Fungal databases	http://nt.ars-grin.gov/fungalatabases/Index.cfm
Mycology Online	http://www.mycology.adelaide.edu.au/
Interactive Diagnostic Key to Plant Parasitic, Freelifving and Predaceous Nematodes	http://nematode.unf.edu/key/nemakey.htm
NemaPIEx	http://plpnemweb.ucdavis.edu/nemalex/index.htm
<i>General</i>	
Crop Protection Compendium	http://www.cabi.org/compendia/cpc/index.htm
Pocket Diagnostic	http://pdiag.csl.gov.uk/products/
Plant Diagnostics Database	http://www.co.missoula.mt.us/extension/plantdata/index.html
Diagnosis	http://www.diagnosis.co.nz/
Diagnostic Keys to Major Tree Fruit Diseases	http://www.caf.wvu.edu/keameysville/wvufarm6.html

Selected references

- Bridge P, Jeffries P, Morse D R & Scott PR (Eds.) 1998 *Information Technology, Plant Pathology and Biodiversity*, CAB International, Wallingford, UK. 478pp.
- Gaston K J & O'Neill M A 2004 Automated species identification: why not? *Philosophical Transactions of Royal Society of London B Biological Sciences*. 359 : 655.

Plant quarantine – means and methods of detection and exclusion of plant pathogens

N Sathyanarayana & S Latha

The main role of plant quarantine is to prevent the introduction and spread of exotic / invasive pests and diseases likely to affect the agriculture and natural environment. In India regulatory measures are in place since the British Rule. An Act, namely "*The Destructive Insects and Pests Act, 1914*" (DIP Act) was enacted by the British Government to safeguard the natural flora from impact of exotic pests. The DIP Act empowers Central Government to issue notifications to regulate import of plants and plant materials from time to time. After the establishment of WTO-SPS Agreements, globalization of trade has taken place with the removal of quantity restrictions and trade barriers. A new scientific look has been brought into the frame work of International Plant Protection Convention (IPPC). To facilitate safe international agricultural trade, many International Standards for Phytosanitary Measures (ISPMs) have been formulated by IPPC to globalize harmonization, transparency and scientific basis of plant quarantine activities. In the last one decade after the establishment of WTO-SPS Measures, the agricultural trade has witnessed tremendous increase in the inflow as well out flow of plants and plant materials in India. This has increased the risk of free flow of plant pests across the globe.

In the past there are several pathogens that had entered into India and have caused and still causing economically perceptible damage to the native crops. To site a few, coffee rust (*Hemilia vastatrix*), coffee berry borer (*Hypothenemus hampei*), Coconut eriophid mite (*Aceria guerreronis*), flag smut of wheat (*Urocystis tritici*), downy mildew of grape (*Plasmopara viticola*), leaf spot of sorghum (*Phyllachora sorghi*), powdery mildew of rubber (*Oidium heveae*), potato wart (*Synchytrium endobioticum*), potato cyst nematodes (*Globodera pallida*, *G. rostochiensis*), downy mildew of sunflower (*Plasmopara halstedii*), bunchy top of banana, peanut stripe virus, bacterial blight of Anthurium (*Xanthomonas dieffenbachiae*) and weeds such as *Parthenium hysterohorus*, waterhyacinth (*Eichhornia crassipes*), *Lantana camara* and Mexican poppy (*Argemone mexicana*) got introduced

and acclimatized with different agro-climatic zones and harming the ecosystem. Still India with its various agro-climatic zones is fortunate to be free from major devastating pathogens such as dwarf bunt of wheat (*Tilletia controversa*), coffee berry disease (*Colletotrichum coffeanum* var. *virulens*), powdery rust of coffee (*Hemileia coffeicola*), Moko disease of banana (*Ralstonia solanacearum* Race 2), South American Leaf Blight (SALB) of rubber (*Microcyclus ulei*), lethal yellowing of coconut (phytoplasma), cadang-cadang of coconut (viroid), swollen shoot disease of cocoa (virus), Fiji disease of sugarcane (virus), soybean downy mildew (*Peronospora manshurica*), soybean cyst nematode (*Heterodera glycines*) etc to name a few.

To safeguard agricultural, horticultural and forestry plants from exotic pests the Government of India notified, "**Plant Quarantine (Regulation of Import into India) Order, 2003**" (PQ Order) under section 3 of **The Destructive Insects and Pests Act, 1914**. This Plant Quarantine Order is operative from 1st January, 2004 and is based on scientific principles of Pest Risk Analysis. This PQ Order replaces **The Plants, Fruits and Seeds (Regulation of Import into India) Order, 1989**. The salient features of the PQ Order, 2003 are:

- ❖ Port of entry is notified for consumption and propagation purposes. The seeds and plants for propagation can be imported only through Amritsar, Chennai, Kolkata, Mumbai and New Delhi. PQ Order also specifies seeds and plant materials that are prohibited, restricted and permitted to import under Schedule IV, V and VI respectively.
- ❖ Import of major plantation crops are prohibited from certain countries due to incidence of destructive pathogens in Schedule IV.
- ❖ Import of major commercial and plantation crops is restricted under Schedule V and is permitted only with the recommendation, supervision, monitoring and testing by the concerned national research institutes established under ICAR.
- ❖ Seeds and plants of vegetables, ornamentals and flowers are regulated under Schedule VI, wherein import is permitted after conducting detailed Pest Risk Analysis (PRA) and specifying various additional declarations for freedom from pathogens of quarantine concern and special conditions to meet import quarantine requirements.
- ❖ Import of seeds of coarse cereals, pulses, oil seeds, fodder and seeds or stock material of fruit plants for propagation is only permitted based on recommendation of EXIM committee of Department of Agriculture and Cooperation. However, small quantity for in-house

or trial purpose is permitted under Schedule XII.

- ❖ Import of live plants is subject to Post Entry Quarantine (PEQ) for specified period under confined conditions. The PEQ facilities to be established by the importer as per the conditions laid down by Plant Protection Adviser to Govt. of India and the same are to be certified by Inspection Authority (IA) listed in Schedule XI. The Inspection Authority shall conduct inspection at regular interval for occurrence of any quarantine pathogens and accord release of the material accordingly.
- ❖ Import of tissue cultured plant materials is encouraged as safe mode of import with additional declarations for freedom from viruses or certification that the tissue cultured plants are obtained from mother stocks tested for freedom from viruses.
- ❖ Thirty one Quarantine weed seeds are notified in Schedule-VIII. All the seeds/ plants/ plant materials imported into India should be found free from these weeds.

Import of seeds/ plants/ plant materials for propagation

The Directorate of Plant Protection, Quarantine and Storage has been entrusted to implement the regulations issued under DIP Act, 1914 to Plant Quarantine Stations situated at international airports, land frontiers and seaports for commercial exchange (import and export) of plants and plant materials whereas National Bureau of Plant Genetic Resources, New Delhi is entrusted with the implementation of regulations on exchange of germplasm materials. The plant materials imported into India for propagation purpose has to undergo plant quarantine checks at three different stages, namely, pre-entry quarantine inspection, port of entry inspection and post-entry quarantine inspection.

Pre-entry quarantine requirements: For consignments of seeds/ plants/ plant materials for propagation, import permit and phytosanitary certificates are mandatory. The importer has to obtain the import permit before import and a copy of the import permit has to be sent to the exporter to comply for freedom from quarantine pathogens as per additional declarations and special conditions stipulated in the import permit and the same should be endorsed in the Phytosanitary Certificate issued at the country of origin. The tissue cultured plants should be free from viruses and / or the inspection of mother crop for freedom from viruses should be carried out and the same to be specified in the Phytosanitary certificate issued at the country of origin.

Port of entry requirements: The Regional Plant Quarantine Stations at Amritsar, Chennai, Kolkata, Mumbai and New Delhi are adequately equipped with state-of the art laboratory facilities and with experts in the field of plant protection. All the consignments of seeds/ plants/ bulbs/ rhizomes/ budwoods/ cuttings and tissue cultured materials for commercial purpose shall be inspected by the plant quarantine officials at the port of entry and shall carry out appropriate testing to detect plant pathogens. Perishable plant materials such as live plants, bulbs, cuttings, saplings, rhizomes, budwoods, etc., intended for propagation are inspected randomly at port of entry and if found free of pests of concern, such materials are given provisional clearance to grow under confined post-entry quarantine facilities, already established by the importer. The plant materials when found infected/ infested with pests of quarantine concern, such materials shall be either deported back to country of origin or destroyed. In case of seeds, detailed laboratory testing will be carried out before release. The release will be accorded when the consignment is found free from live infection, if found infected the same shall be treated before release. In case of interception of quarantine pathogens, the consignment shall be deported or destroyed by incineration.

Post-entry quarantine requirements: The live plant materials are perishable in nature and are the more important means of carrier of plant pathogens, they are not accorded immediate release and instead they are permitted to be grown in a confined post-entry quarantine area wherein the imported plants are grown in isolation. The importer should establish post-quarantine facility to grow the imported plants till the stipulated period as mentioned in Schedule-VI. The PEQ facility has to be certified by Inspection Authority (Head, Department of Plant Pathology, State Agricultural University) and the plants has to be grown under the supervision of IA, inspected at regular interval till final clearance is granted.

Methodology

The samples subjected for inspection are true seeds, saplings, rooted plants, cuttings, rhizomes, bulbs, suckers, budwoods, unrooted cuttings, nuts and tissue cultured plants. The plants and seeds at the port of entry are inspected and subjected to washing test, blotter test, grow-out test, Agar plate, ELISA and Electron Microscopy, depending upon the nature of pathogen anticipated/ targeted.

Visual observation: All the seed samples are first examined visually under magnoscope for the presence of symptoms such as discolouration, malformation, encrustation and deformation.

Microscopic observation: In case of fungal infections, where sporulation / fruiting bodies are visible to the naked eyes, direct microscopic observations are carried out. Eg. downy mildew of soybean, smuts, rusts, bunts, etc. In case of bacterial infections, a ooze test is carried out to ascertain the presence of bacterium in the plant tissue.

Blotter test: The seeds are plated as per ISTA norms a minimum of 400 seeds is plated per variety in seedling germination boxes. After 7 days incubation at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ under alternating cycles of 12 h light and darkness, the seeds are examined for signs of fungal sporulation/ bacterial symptom on the cotyledons/ primary leaves. One hundred seeds per variety are surface sterilized with 1% sodium hypochlorite to suppress the growth of surface bound contaminants and to observe exclusively for seed-borne or seed-transmitted fungi. In case of live plants, the infected area is cut into small pieces, surface sterilized with 1% sodium hypochlorite for 3 min and plated on moist blotter and incubated at alternative cycles of light and dark at $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 3 to 7 days for fungal sporulation.

Washing test: About 2000 seeds/ 50-100 g of seeds are soaked in sterile distilled water for an hour in conical flask. A few drops of Tween 20 is added as wetting agent/ detergent. The conical flasks are gently rotated in Wrist Action Shaker for 10 minutes. The resulting suspension is concentrated by low speed centrifugation and the pellet is re-suspended in few drops of 50% Glycerol and examined under compound microscope for the detection of rusts, smuts, bunts, downy mildews, powdery mildews. To detect the viability of oospores, few drops of 0.05 or 0.1% of Phloxine B (aqueous solution in deionized water) is added to the oospore pellet after centrifugation and incubated at room temperature for 20min.

Grow-out test: About 100 to 200 seeds are sown 2-3 cm deep in sterilized soil/ coir peat in a green house/ controlled environment chamber. Germinated seedlings are observed regularly for symptoms of viral / bacterial diseases upto 21 to 30 days

Agar plate for fungal infections: Water agar: To detect latent infection in seeds, few seeds are plated on water agar in Petri plats or tubes and germinated seedlings are examined for appearance of pathogens and disease. The seeds are surface sterilized by immersion in 1% Sodium hypochlorite for 5 min. to remove surface contaminants before plating. In case of live plants, the infected area is cut into small pieces, surface sterilized with 1% sodium hypochlorite by immersion for 3 min and washed with sterile distilled water by immersion for 3 min and plated on Water agar. After 2 - 3 days of incubation, a portion of the fungal mycelial filaments along with agar is

removed by stubbing and plated on PDA and incubated under alternate cycle of light and dark at 25°C for 4 – 5 days.

Agar plate for bacterial infections: The bacteria observed by ooze test, are isolated by streaking on to agar plates of differential or selective media. The selected tissue is surface sterilized by immersion in 1% sodium hypochlorite for 3 minutes and rinsed in sterile distilled water for 3 minutes. The tissue is then macerated on sterile glass slide in a drop of sterile water. A loop full of bacterial suspension from the infected plant / seed material is streaked on to differential/ semi-selective/ selective media and incubated.

DAC-ELISA test: The suspected seed/ leaf samples are subjected to DAC-ELISA by using polyclonal/ monoclonal antibodies (antisera). The ground seed/ plant extract of suspected viral infected sample is coated in the microtitre plates, polyclonal/ monoclonal antibodies are added and the antigen-antibody reaction is visualized by using an enzyme and suitable substrate to form a coloured product. With the help of an ELISA Plate Reader, the reaction is read at 405 or 650 nm, then recorded and scored whether it is positive or negative.

Transmission electron microscope: Through simple leaf dip preparation, the infected tissue is macerated on a clean glass slide by fine chopping with a sterile blade or slightly macerated with the tip of a glass rod, few drops of Phosphate buffer is added to the sap. 10µl of sap is placed on a piece of parafilm and the carbon coated copper grid is placed (floated) on top of the drop (carbon coated side facing the drop). After few minutes (5 – 10 min) the grid is washed with sterile distilled water in drop-by-drop manner and then stained with few drops of 2% Uranyl acetate/ Phosphotungstat negative stain in drop-wise manner. The grid is allowed to air dry for few minutes before observation under Transmission Electron Microscope. When the virus concentration is very less, ISEM is also employed to trap the virus particles. Based on the morphological characters, the viruses are grouped and identified.

By carrying out one or combination of different detection techniques as mentioned above, the pathogens are intercepted and identified. The quarantine pests are the major target organisms for which detailed tests are carried out; whenever quarantine pests are intercepted the same is also sent to IARI for confirmation of identification. The consignments found infected with non-regulated quarantine pests are treated appropriately before according release. The consignments wherein the quarantine pests are intercepted the same are deported or destroyed by incineration. On several

occasions quarantine pests have been intercepted in the past. To cite a few, *Agrobacterium tumefaciens* on Rose plants from Netherlands (Sathyanarayana *et al.* 2002), *Xanthomonas campestris* pv. *dieffenbachiae* on Anthurium from Netherlands (Sathyanarayana, 1998), *Erwinia chrysanthemi* on Carnation from Spain and Dendrobium from Thailand & Singapore, *Pseudomonad cichorii* on Cichorium seeds from Netherlands, *Pseudomonas syringae* pv. *maculicola* on Cabbage seeds from USA, Poty virus in Oil Palm seedlings raised in PEQ facility, imported from Costa Rica (Reddy *et al.* 1996), Cymbidium mosaic virus and Odontoglossum mosaic virus on Dendrobium plants from Thailand (Sathyanarayana *et al.* 2003), Frangipani mosaic tobamo virus on Plumeria from Malaysia, *Embellisia allii* on Garlic bulbs imported from China, (Latha *et al.* 2006) *Fusarium oxysporum* f.sp. *callistephi* on Marigold seeds from Mexico, *Claviceps purpurea* on Wheat from Turkey & Canada, *Coleosporium domingense* on Plumeria from Malaysia, *Drechslera maydis* on Maize from Philippines & Thailand, *Peronospora farinosa* on Beet seeds from USA, *Peronospora manshurica* on Soybean seeds from USA, *Phytophthora megasperma* on Soybean seeds from USA, and *Plasmopara halstedii* on Sunflower from USA, South Africa & Germany.

Plant quarantine acts as a filter than as a barrier in international agricultural trade. The stringent phytosanitary measures / regulations are in place to keep the exotic enemies away. Prevention is better than cure and the same holds good in plant quarantine that prevention is better than control or management options once the exotic pathogens enters into India. The urge to get high yielding varieties for profit as well as to meet the domestic need has paved way for bulk import of seeds and plants and this has opened the gate for entry of exotic pathogens accompanying the propagative materials into India, to mitigate the need and the accompanying pest problem, following the plant quarantine regulations are the only way to safeguard agricultural, horticultural and forest plants from exotic/ invasive pests. To implement safe quarantine regulations, the 5 regional stations at Amritsar, Chennai, Kolkata, Mumbai & New Delhi are strengthened with experts in the field of plant protection and hi-tech laboratory facilities. When a quarantine pest is repeatedly intercepted from particular origin on specific commodity, import of such consignments from the particular country is banned. Tissue cultured plants are considered far safe way of import for propagation than true seeds or live plants. Vigilant and rapid quarantine screening is the only way to safeguard our agricultural, horticultural and forest plants from exotic pathogens. The details with regard to import and

export of plant and plant materials can be obtained from
www.plantquarantineindia.org

Selected references

- Latha S, Sathyanarayana N & Reddy O R 2006 In: *Proc. of National Symposium on Emerging Plant Diseases, their Diagnosis and Management*, 31 January - 2 February 2006, West Bengal University. P78.
- Reddy O R, Sathyanarayana N, Vani S, Babu D V N & Latha S 1996 In: *National Seminar on Oil palm Production and Processing*. 13 - 14 February 1996, Mysore, Karnataka, India. pp. 28 - 31.
- Sathyanarayana N, Reddy O R, Latha S & Rajak R L 1998 *Plant Disease* 82(2) : 262.
- Sathyanarayana N, Latha S & Reddy O R 2002 In: *Proc. of National Seminar on Resources Management in Plant Protection During Twenty First Century*, November 14-15, 2002, Hyderabad, India. pp. 177 - 179.
- Sathyanarayana N, Latha S & Reddy O R 2003 *Indian Journal of Plant Protection* 31(2): 118 - 119.

V

BIOCONTROL

Isolation, identification and evaluation of fungal biocontrol agents

B Rananujam

Fungal biocontrol agents for plant disease management

Though biocontrol agents include all classes/groups of organisms existing in an ecosystem, maximum emphasis for developing biocontrol programmes was given to fungal and bacterial agents primarily because of ease of their mass multiplication and formulation. The important species fungi studied as biocontrol agents are *Trichoderma viride*, *T. harzianum*, *T. virens*, *Chaetomium globosum*, *Coniothyrium minitans*, *Sporidesmium sclerotivorum*, *Ampelomyces quisqualis*, *Aspergillus niger* and saprophytic yeasts. Among the potential biocontrol fungi, species of *Trichoderma* have received maximum attention mainly due to high efficacy, broad spectrum of activity, and ease in isolation and cultivation. In recent years successful use of fungal biocontrol agents like *Trichoderma* spp. for the control of soil borne diseases in several crops have been reported. Among these, *T. viride*, *T. harzianum*, *T. virens* are under intensive research because of their wide natural occurrence, biocontrol potential against fungal and nematode diseases. The biocontrol agents provide protection against plant diseases either by direct action against the pathogen by mycoparasitism and antibiosis and indirectly by competing the pathogen for nutrients, oxygen or space. Apart from this, *Trichoderma* spp. also exhibit plant growth promoting activity in several crops. There are several formulated products of these agents are already available or are in the process of development.

Isolation of *Trichoderma* species

Trichoderma spp. are commonly present in soils (rhizosphere as well as Non-rhizosphere and rhizoplane), decaying organic matter, manure heaps, crop residues.

Collection of soil samples for bioagent isolations

Soil samples should be collected from a field where the pathogen is known to be present but disease occurrence is low. Areas where a pathogen was introduced but not established, and areas of monoculture of crops where diseases intensity has decreased after a few years with a susceptible crop

provides excellent chances of finding a suitable antagonist. Soils of special interest also should be included. In certain cases where the history of the field and disease occurrence is not available, then biologically active soils that contain a diverse population of microorganisms should be sampled. Soil should be collected to a depth of 15 cm in the upper surface including rhizosphere and rhizoplane samples. The collected samples should be analyzed as soon as possible. For isolation of phylloplane micro flora, healthy foliar parts (like flowers, fruits, leaves) from diseased plants should be collected.

Isolation from rhizosphere (Dilution plate method)

1. Separate rhizosphere soil from 5-6 roots with the help of a brush in a petri plate.
2. Add 10 g of rhizosphere soil in 100-ml sterile water blank and shake it for 15 minutes on a magnetic shaker.
3. Prepare serial dilutions 10^{-2} to 10^{-6} .
4. Transfer 1 ml each of dilution 10^{-4} to 10^{-6} to sterile petri plates.
5. Pour melted and cooled *Trichoderma* specific agar medium
6. Incubate the plates at 25 °C for 5-7 days.
7. Transfer isolated colonies into PDA slants.

Isolation from rhizoplane

1. Take root pieces from the first dilution in screw cap bottle with 100 ml sterile water and shake it well.
2. Serial washings (10-20) are given to the above roots with sterilized water until clear root surface is exposed.
3. Plate the washed roots on specific medium.
4. Incubate the plates for 5-7 days at 25 °C.

Isolation from phylloplane

1. Collect fresh, healthy leaves (of all ages) in sterile polythene bags.
2. Cut five discs each of 6-mm diameter from every leaf using sterile cork borer.
3. Transfer 50 disks to 100-ml water blank and stir for 20 minutes using magnetic stirrer.
4. Prepare serial dilutions (10^{-1} and 10^{-2}).
5. Transfer 1-ml aliquot from all dilutions to sterile Petri plates and pour specific agar medium.

6. Incubate the plates for 5-7 days at 25 °C for obtaining colonies of antagonistic fungi.

Composition of specific media

1. *Trichoderma specific medium (TSM) (Elad et al. 1981)*

Mg SO ₄ . 7 H ₂ O	0.2 g
K ₂ HPO ₄	0.9 g
KCL	0.15 g
NH ₄ NO ₃	3.0 g
Glucose	3.0 g
Chloromphenicol	0.25 g
Fenaminosulf	0.3 g
PCNB	0.2 g
Rose Bengal	0.15 g
Water	1 L

2. *Improved TSM (Elad & Chet 1983)*

Mg SO ₄ . 7 H ₂ O	0.2 g
K ₂ HPO ₄	0.9 g
KCL	0.15 g
NH ₄ NO ₃	3.0 g
Glucose	3.0 g
Chloromphenicol	0.25 g
Fenaminosulf	0.3 g
PCNB	0.2 g
Rose Bengal	0.15 g
Benomyl	0.02 g
Captan	0.2 g
Water	1 L

3. *Adapted selective medium for Trichoderma spp. (Askew & Laing 1993)*

Mg SO ₄ . 7 H ₂ O	0.2 g
K ₂ HPO ₄	0.9 g
KCL	0.15 g

NH ₄ NO ₃	3.0 g
Chloromphenicol	0.25 g
PCNB	0.2 g
Rose Bengal	0.15 g
Captan	0.2 g
Propamocarb	1.2 ml
or	
Metalaxyl	1.6 g
Water	1 L

Estimation of *Trichoderma* population in soils

Collect the soil sample from the field, mix well and made into fine particles. Collection of soil sample should be made in the root zone at 5-15 cm depth preferably from rhizosphere.

Materials required for isolation/estimation of *Trichoderma* population

1. Sterilized water 100ml in conical flasks (250 ml)
2. Sterilized water 9ml in test tubes
3. Sterilized pipettes 1ml
4. Sterilized petridishes
5. *Trichoderma* selective medium (TSM)

Procedure

1. Ten grams of soil sample suspended in 90ml of sterile distilled water (1:10 or 10⁻¹). Shake well.
2. Take one ml from this and transfer to 9ml of sterile water in tube (1:100 or 10⁻²).
3. Make serial dilutions by transferring 1ml of the suspension to the subsequent tubes to get 10⁻⁶ to 10⁻¹⁰.
4. The dilutions generally followed for isolation and estimation in soil are 10⁻¹ to 10⁻⁴ and for estimation in formulations are 10⁻⁴ to 10⁻¹⁰
5. Transfer one ml of the desired soil suspension to petriplates containing TSM. Rotate the plate gently. Incubate at room temperature. Observe the development of colonies.

Observations

Trichoderma colonies on the selective medium will be white initially and later on turn to green.

Count the number of colonies developing in individual plates.

Calculations (for quantitative estimation)

Assume average number of *Trichoderma* colonies per plate is 4 at 10^{-4} dilution (1:10,000)

1ml of 1: 10,000 dilution contains : 4 colonies

1gm of soil/formulation will contain : $4 \times 10,000 = 40,000$ colonies
 $= 4 \times 10^4$ colonies

Identification of *Trichoderma* species

Trichoderma Pers. ex Fr., is a genus under deuteromycotina, Hyphomycetes, Phialosporae, Hyphales, Dematiaceae. Various species under these genera can be identified by morphological characters. Macromolecular approaches (Enzyme and Nucleic acid analyses) have been given importance nowadays because lot of confusion still existing in presently available taxonomy of *Trichoderma*. Morphological characters used in specific recognition in

have been outlined by Rifai (1969) and Bissett (1984, 1991a-c, 1992). Rifai (1969) differentiated *Trichoderma* from *Gliocladium* primarily by the angle at which branches and phialides were borne. In *Gliocladium* branching is penicillate or convergent with phialides closely appressed. The branching pattern can be seen in figures.

Morphological characters for identification of important species of *Trichoderma*

T. viride Pers.: Fr. ex S.F. Gray

Conidia : 1-celled, green or brownish, subglobose to ellipsoid, rough-spored

Conidiophores : Narrow, flexuous, primary branching at regular intervals, paired or in whorls of three, short and not extensively branched

Phialides : Mostly in verticils of 2 or 3

Chlamydospores : present in most isolates

Colony characters : Grows rapidly upto 9 cm diameter after 3-4 days at 25 °C, Colonies Fluccose

Reverse pigment : Colourless to dull yellowish

T. harzianum Rifai

Conidia: Subglobose to obovoid or short ellipsoid, 1.7-3.2 x 1.3-2.5 μm (avg. 2.4 x 1.9 μm)

Conidiophores: Hyaline, smooth walled, straight or flexuous up to 8 μm wide near base, 2.5-4.5 μm wide for most of the length, highly branched, primary branching at right angles, whorls of 2 or 3, secondary branching in whorls of 2-4, ultimate branch 1 celled, 3.5-10 x 2.5-6 μm .

Phialides: Ampuliform to subglobose or lageniform, 3.5-7.5 x 2.5-3.8 μm , arise mostly in crowded and diverse whorls of 2-6.

Chlamydospores: Fairly abundant, intercalary and terminal, 4-12 μm

Colony characters: Grows rapidly, floccose aerial mycelium, white to grayish or rarely yellowish

Reverse pigment: Colorless to dull yellowish

***T. hamatum* (Bon.) Bain.**

Conidia: oblong to ellipsoid, dull green, 3-4.5 x 2.1-2.8 μm (avg. 3.9-2.5 μm)

Conidiophores: 5-10 μm diameter near the base, highly branched, primary branches relatively short, usually in whorls of 2-5, highly rebranched, ultimate branch one celled, barrel-shaped or short cylindrical, mostly 3.5-7 x 3-5 μm

Phialides: subglobose to ellipsoidal or ampulliform, 3.3-5.6 x 2.8-3.5 μm , arise in crowded whorls of 3-6.

Chlamydospores: abundant, 3.3 x 1.8 μm

Colony characters: colonies grow moderately rapidly, limited aerial mycelium is floccose, white to grayish

Reverse pigment: Colorless to pale- greenish yellow

***T. virens* (Miller, Giddens & Foster) von Arx = *Gliocladium virens* Miller, Giddens & Foster**

Conidia: Broadly ellipsoidal to obvoid, 3.5-6.0 x 2.8-4.1 μm (avg. 4.4 x 3.5 μm), dark green, conidia from adjacent phialides often coalescing into large globoid masses

Conidiophores: conidiophores sub hyaline, 30-300 μm long, 2.5-4.5 μm in diameter, towards base frequently unbranched for about half of the length, towards the apex branching irregularly with each branch terminated by a cluster of 3-6 closely addressed phialides, branches arise at right angles or refluxed towards the apex, primary branches arising singly or in opposite

pairs immediately beneath the septa, rebranched irregularly once or twice, ultimate branches one or two celled

Phialides: Ampulliform to lageniform, 4.5-10 x 2.8-5.5 μm , swelling in the middle, mostly arising in closely appressed verticils of 2-5 or terminal branches

Colony characters: grows rapidly, aerial mycelium floccose, white to grayish (**Plate 3 and 4**)

Reverse pigment: colourless

1.4 Identification services in India

Identification services for fungi are available in the institutes mentioned below.

- Division of Plant Pathology, Indian Agricultural Research Institute, Pusa, New Delhi-110 012
- Institute of Microbial Technology (CSIR), Chandigarh
- Fungal Identification Service, Agharkar Research Institute, Pune

Screening and selection of potential antagonists of plant pathogens

***In vitro* evaluation of fungi for antagonistic effect on plant pathogens:**

Evaluation of test fungi for their antagonistic effect on plant pathogenic fungi can be carried out by (i) Dual culture method, (ii) Antibiosis test for production of diffusible inhibitory metabolites by candidate fungi effective against pathogens, and (iii) Antibiosis test for production of volatile compounds by candidate fungi inhibitory to pathogens. In the dual culture method, the overall ability of the test fungus in inhibiting the pathogen through competition for nutrients, production of inhibitory compounds and hyper parasitism was tested. Antibiosis tests were used to determine the ability of the test fungus to produce diffusible (non-volatile) and volatile metabolites effective against the pathogen.

Dual Culture Method: Direct opposition method using simultaneous inoculation of pathogen and opposing fungus as described by Webber and Hedger (1986) was adopted. Six mm discs of pathogen and test fungus (antagonistic fungus) cut from 5-7-day old cultures were placed on 2% Malt agar plates (90 mm diameter) in such a manner that they lie opposite to each other at the corners of plate. Plates inoculated with either the pathogen alone or test fungus alone in a similar position served as control. Three replications can be used in each case and the plates incubated for 5-15 days. After 24 hr of incubation, radial growths of pathogen and test fungus were measured in dual and monoculture plates. These measurements

can be used to calculate the percentage of inhibition of pathogen/ test fungus. Overgrowth of pathogen/test fungus, if any may also be recorded. Attempts may also be made to re-isolate pathogen/ test fungus (viability test) from the interaction sites by transferring 6 mm discs cut from the interaction zone/sites to fresh PDA/ MA plates and examining the renewed growth. Based on the observations taken in dual cultures, the type interaction occurring between pathogen and test fungus may be identified using the key developed by Webber and Hedger (1986) consisting of the following types of interactions:

- (i) Intermingling category (I): Intermingling of colonies without macroscopic / microscopic interaction.
- (ii) Overgrowth category (O): Overgrowth on pathogen colony by opposing fungus usually accompanied by the inhibition of pathogen colony on or after contact.
- (iii) Mutual inhibition with pigmental band (MP): Mutual inhibition of pathogen and opposing fungus with a pigmented band of mycelium at the junction.
- (iv) Mutual inhibition with a clear zone (MC): Mutual inhibition of pathogen and opposing fungus with a clear zone between the colonies.
- (v) Extreme inhibition of pathogen (E): Extreme inhibition of pathogen at distance of 5 mm or more and little or no reciprocal inhibition of the opposing fungus.

In the above tests, as the two organisms grow towards each other, the reduced growth rate of the pathogen at a distance from the periphery of the potential antagonist indicate production of an "antibiotic". Other reactions that can occur in dual culture are that both organisms stop growing upon contact, with a small but clearly marked space between them. Antagonism between two organisms also is indicated when the pathogen stops growing upon contact with the antagonist and its mycelium begins to lyse backwards and the antagonist may continue its growth over the colony of the test fungus (Mycoparasitism). Microscope. Hyphal interaction in dual culture is also can be studied by growing the fungi on a cellophane membrane in petri dish with water-agar plus glucose (0.5% w/v). After 4 to 5 days, pieces of cellophane membrane from the contact zone are cut and prepared for microscopic observation. Antagonism also is indicated when the aerial fluffy mycelium of the pathogen is appressed when growing near the antagonist.

Antibiosis test for production of diffusible inhibitory metabolites by the

test fungi against pathogen: The test was carried out using cellophane paper method described by Dennis and Webster (1971a). For this, cellophane paper (50 μm thick) discs of 90 mm diameter were cut and sterilized in an autoclave at 121°C for 15 minutes and then each sterilized disc was aseptically placed over 2% Malt agar medium taken in a Petri plate (90 mm diameter). Six mm discs of each candidate fungus in three replications may be placed at the center on the cellophane paper and incubated for 2-5 days. After this, the cellophane paper and the adhering fungus may be removed carefully and a six mm disc of pathogen immediately placed on the medium at a central position previously occupied by the candidate fungus. The radial growth of pathogen is recorded after every 24 hr and compared with the growth in control plates. Based on this, the percentage inhibition of pathogen, if any, was calculated.

Antibiotics in culture filtrate: In this method the antagonistic fungus is grown in a suitable liquid medium in stationary or shake culture. After sufficient growth has occurred mycelium and other cells are removed by filtration through filter paper and then sterilized by passing through sintered glass filter. The sterile filtrate in desired concentration is added to sterile culture medium and plated and the pathogen is inoculated at the centre of the plate. Antagonist grown on PDA not amended with filtrate serves as control. Reduction in colony growth in filtrate-amended medium compared to control indicates secretion of antibiotic compounds into culture by antagonistic fungi.

Antibiosis test for production of volatile inhibitory compounds by candidate fungi effective against pathogen: The test was carried out by slightly modifying the sealed Petri plate technique described by Dennis & Webster (1971 b). For this, two Petri dish bases (90 mm) containing 20 ml of solidified 2% Malt agar medium were sealed together by an adhesive tape and the candidate fungus (antagonistic fungus) was grown in the bottom plate for one, three, six and 10 days at $25 \pm 2^\circ\text{C}$ and allowing the medium on the upper plate at the same time to adsorb any volatile compounds produced, if any, by the candidate fungus. The upper Malt agar plates after the required exposure to the candidate fungus were carefully removed and inoculated with pathogen and the radial growth of pathogen was recorded after every 24 hr and compared with the growth in control plates. Based on this, percentage inhibition of pathogen, if any, can be calculated.

In vivo methods of screening

Greenhouse pot tests and field tests : As *in vitro* methods are not dependable, greenhouse and field studies should be carried out for identifying potential antagonists. The effect of the antagonistic fungi on the survival, saprophytic growth, and parasitic activity of the pathogen in soil may be tested. The soil used for greenhouse and field studies should be suitable for the growth of the pathogen and antagonist. Where ever possible, use different types of soils collected form different agro-climatic regions for both laboratory and greenhouse studies. To obtain meaningful data, the quantity of the propagules of the pathogen and antagonist added to per unit volume, weight, or area of the soil should be controlled. Antagonists can be applied by way of seed treatment, soil amendment or as foliar sprays (for control of foliar diseases).

Seed treatment is followed for control of soilborne pathogens, which incites seed decay, damping-off, root, rot and wilt diseases. Spore and mycelial suspension of antagonistic fungus obtained from culture plates or from liquid broth and mixed with 1% carboxymethyl cellulose or gum arabic and coated to the seeds. The treated seeds are sown in pathogen-infested soils (Greenhouse or field) and observations on disease incidence are recorded at regular intervals. Antagonistic fungi can also be grown on cheap organic substrates and amended to pathogen-infested soils. Pathogen and antagonists can be incubated for 10 to 15 days after amending to the soil to give antagonist sufficient time for parasitizing pathogen propagules.

Fungi having antagonistic potential against a wide range of plant pathogens can be identified by following various screening procedures described earlier. As specificity does occur with antagonistic fungi also, suitable isolates of these fungi effective against a specific pathogen in a particular crop or a location need to be identified.

Entomofungal pathogens for crop pest management

Insects like other organisms are susceptible to a variety of diseases caused by viruses, bacteria, fungi, protozoans, rickettsia, mycoplasma and nematodes. Fungi which infect insects are called entomopathogenic fungi and the fungal diseases on insects are referred as mycosis. Entamopathogenic fungi play an important role in the natural pest control in various crops through epizootics. Fungal control of insects refers to the exploitation of these disease-causing fungi to reduce the pest population below the damaging levels through introduction and augmentation. Entamopathogenic fungi are gaining importance in the crop pest control in recent years, although *Bacillus thuringensis* (*B.t*) and Nucleopolyhedroviruses (NPVs) are the most widely used microbial agents at present. Fungal pathogens have certain advantages in pest control

programmes over other insect pathogens. Mass production techniques of fungi are much simpler, easier and cheaper than those used for *B.t* and NPVs. Fungi unlike bacteria or viruses directly infect through insect cuticle and do not require ingestion for infection and so sucking insects are also infected by entomopathogenic fungi.

More than 750 sp. of fungi, mostly deuteromycetes and entomophthorales from about 100 genera are pathogenic on insects, many of them offer great potential for pest management. Species that have been most intensively investigated for mycoinsecticides in the crop pest control include *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii*, *Hirsutella thompsonii*, *Nomuraea rileyi*, *Paecilomyces farinosus* etc. The first two species have been used in large scale for the control several crop pests over a number of years, while others have been attempted in recent times. Several commercial formulations based on these fungi are produced in different countries .

Most of the entomopathogenic fungi infect the host through the cuticle. The process of pathogenesis begins with adhesion of fungal spore on cuticle followed by germination, penetration and development of fungus inside the host leading to the death of the host. Fungi usually cause insect mortality by nutritional deficiency, destruction of tissues and releasing of toxins. After the penetration of germinating hyphae into the haemocoel, the fungus produces hyphal bodies, hyphal strands and protoplasts that fill the haemocoel completely. Several mycotoxins like, Beauvericin, Beauverolides Bassianolide (by *B.bassiana*, *V.lecanii*, *Paecilomyces* spp.) and Destruxins A, B, C, D, E, F (by *M.anisopliae*) are produced during pathogenesis and these act like poisons for the insects. After the death of the insects the fungus breaks open the integument and forms aerial mycelia and sporulation on the cadavers.

At early stages of fungal infection, the insects show little or no symptoms except for a few necrotic spots that may develop at the invasion sites. At late stages of infection, the insects become restless, less active, loose appetite and coordination. The internal tissues show disintegration prior to the insect death. External fungal growth on the cadavers is another diagnostic feature of the fungal infection in insects. An insect covered with powdery white spores would be suspected of having *B.bassiana* infection, powdery green spores would suggest an infection with *M.anisopliae* or *N.rileyi* and yellowish spores would indicate having *P.farinosus* or *Aspergillus flavus* infection. The fungal infected insects are usually hardened and not soft like in bacterial infections.

Environmental conditions particularly humidity and temperature

plays an important role in the infection and sporulation of entomopathogenic fungi. Very high humidity (> 90%RH) is required for spore germination and sporulation outside the host. Most of the entomopathogenic fungi in tropical and subtropical areas require an optimum temperature of 25°C for epizootics.

Isolation of entomopathogenic fungi

Collection of fungal infected insects

The best season for collection of fungal infected insects from different crops/regions is monsoon and winter periods (July-January), during which fungal infections on various crop pests are common due to the prevalence of high humidity and low temperature

Characters of fungal infected insects

- i. The most striking characteristic of a fungal infection is the presence of mycelium in or on the affected insect.
 - An insect covered with **powdery white spores** would be suspected of having *B.bassiana* infection
 - An insect covered with **powdery green spores** would suggest an infection with *M.anisopliae* or *N.rileyi*
 - An insect covered with **yellowish spores** would be suspected of having *P. farinosus* infection
 - An insect covered with **greenish yellowish spores** would suggest an infection with *Aspergillus flavus*
- ii. Hemocoel of the fungal infected insects is filled with hyphal bodies
- iii. The fungal infected insects are usually hardened and not soft like in bacterial infections.
- iv. The position of the fungal infected insect may be fixed to the leaves/plant part or relaxed on the ground.

Isolation of entomopathogenic fungi from the infected insects

The fungus can be isolated from the infected insects in two ways, either from the hemolymph or directly from the fungal growth on the surface.

- i. Isolation from hemolymph: Infected insects are first surface sterilized by immersing them in 5% sodium hypochloride solution for a few minutes and rinsed in sterile water three times. Then the specimen is cut open in a sterile plate and a small portion of infected hemolymph/tissue is streaked on PDA (Potato, 200.0g, Dextrose, 10.0g, Agar, 15.0g, and Distilled water,

1000ml).for isolation of *B.bassiana*, *M.anisopliae*, *V.lecanii* and *Paecilomyces* sp. or Sabouraud maltose/dextrose agar with yeast extract medium, SMAY/SDAY (Peptone,10.0g, Maltose/Dextrose, 40.0g, Yeast extract,2.0g, Agar,15.0g, and Distilled water, 1000ml) for isolation of *N.rileyi*. For suppression of contaminating bacteria and for restricting fast growing fungi like *Apergillus*, *Penicillium* etc., antibacterial/fungal chemicals like, Chloramphenical @ 50ppm and rose bengal @ 100ppm may be added to the basal medium. The pure colonies of entomopathogenic fungi may be sub cultured on PDA/SMAY slants and stored in a refrigerator.

ii. Direct isolation from surface of the infected insects: Mycelium and spores can be removed from a fresh specimen and placed directly on the PDA/SMAY/SDAY medium. However, this isolate should be compared with that obtained from infected hemolymph/tissue.

Storage of entomopathogenic fungi

The simplest method of storage is maintenance of cultures on Potato carrot medium (PCA) in slants capped with wax /parafilm under refrigeration or immersed in paraffin oil at room temperature. Many fungi can be stored for several years under lyophilization, liquid nitrogen or in silica gel.

Identification of entomopathogenic fungi

Beauveria bassiana (Bals.)Vuill.

This is known as white muscardine fungus and it has been reported long back to cause disease in silkworms. It occurs worldwide and has one of the largest host list (700 host species) and also occur in soil as a saprophyte. The hosts are mainly in the orders of Lepidoptera, Coleoptera, and Hemiptera and a few in Diptera and Hymenoptera. Some of the major insect pests susceptible to this fungus in India are *Helicoverpa armigera*, *Spodoptera litura*, *Holotrichia consanguinea*, coffee berry borer, rice hispa etc. *B.bassiana* infected cadavers show white aerial mycelia .

Colony and conidial characters of *B.bassiana*

- Colonies white with abundant cottony aerial mycelium.
- Hyphae cylindrical, 3.5 μ wide, hyaline, septate.
- Conidiophores single or branched, abundant, arising from vegetative hyphae, bearing groups of clustered conidiogenous cells
- Conidiogenous cells or Phialides globose to flask-shaped ((3-5 X 3-7 μ m) with well developed rachis upto 20 μ m long and 1-1.5 μ m wide.
- Conidia are borne at thread-like apex of the phialide on a series of

zigzag branchlets, more or less comparable to a cyme.

- Conidia globose(1-4 μ) to oval (1.5-5.5 X 1-3 μ), smooth, hyaline

Beauveria brongniartii

B. brongniartii is found highly suppressive to root grub species like, *H. consanguinia*, *H. serrata* and *Maladera nathani* infesting groundnut, sugarcane, rubber etc. *B. brongniartii* differs from *B. bassiana* by exclusive presence of oval shape spores. Phialids slender, rarely clustered, short, not zig-zag. Conidia oval shaped.

***Metarhizium anisopliae* (Metschn.) Sorokin**

This is commonly known as green muscardine fungus and is widely distributed and recorded on more than 300 insect species. Some of the serious pests susceptible to *M. anisopliae* in India are *Oryctes rhinoceros* of coconut, white grubs (*H. consanguinia*, and *M. nathani*) infesting groundnut, potato, sugarcane etc and cashew stem borer (*Placederus ferrugenus*). *M. anisopliae* infected cadavers show typical green aerial mycelia and sporulation on the surface. The colony of *M. anisopliae* appears white, when young but as conidia mature, the colour turns to dark green (Plate-2). Mycelium composed of hyaline, septate, branched hyphae. Conidiophores short, erect, hyaline, septate, simple or branched, terminating in single or a cluster of phialides. Conidia 1-celled, hyaline, smooth, long-ovoid to cylindrical (4.8 X 1.6 μ), abstricted from the tips of the phialides and forming long basipetal chains/columns (Fig-2). Based on the size of conidia, *M. anisopliae* is divided in to two varieties viz., *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *major*. The former has short conidia, of 3-9 μ length and the latter has long spores of 9-18 μ length.

- Colonies with dark green or olive green conidial masses
- Mycelium composed of hyaline, septate, branched hyphae.
- Conidiophores short, hyaline, septate, erect, simple or branched, terminating in single or a cluster of Phialides.
- Phialide cylindrical to narrow ellipsoid
- Conidia 1-celled, hyaline, smooth, long-ovoid to cylindrical (4.8 X 1.6 μ), often narrowed centrally abstricted from the tips of the phialides forming long basipetal chains/columns.

***Verticillium lecanii* (Zimm.) Viegas**

It is known primarily as a pathogen of aphids, scales, whiteflies, thrips and red spider mites. *V. lecanii* is also a mycoparasite of rust and powdery mildews. The fungus is characterized by the presence of

conidiophores in verticillate whorls and on which conidia are borne in slime or mucus balls .

- Colonies white to pale yellow, cottony
- Conidiophores erect or little differentiated
- Phialides solitary or in scant whorls, aculeate, extremely variable in size, usually 12-40X0.8-3.0 μ m
- Conidia in heads or parallel bundles, cylindrical with rounded tips or ellipsoidal, 2.3-10X1.0-2.6 μ m
- Chlamydospores absent

***Nomuraea rileyi* (Farlow) Samson**

It infects a number of lepidopteran larvae such as *Spodoptera litura*, *S. exigua*, *Helicoverpa armigera*, *H. zea*, *Trichoplusia ni*, *Plusia sp.* and various noctuid defoliators. When favorable conditions of humidity (>70%) and temperature (20-30°C) exist for a long time, *N. rileyi* is known to cause natural epizootic with in larval populations of *S. litura*, *H. armigera* and *Plusia sp.* on castor, cotton, groundnut, redgram and niger in Andhra Pradesh in India

- Colonies slow growing, white initially, later turn pale green to malachite green
- Hyphae 2-3 μ in dia., smooth, septate, hyaline and slightly pigmented
- Conidiophores are long upto 160 μ , forming dense cluster of branches each bearing 2-3 compact phialides, around the stalk.
- Phialides are short and rounded, cylindrical to globose, with very swollen base tapering abruptly to a narrow neck
- Conidia are produced in dry divergent chains on phialides and are elliptical to cylindrical in shape, measure 3-4 X 2-2.5 μ and pale green.

Paecilomyces sp.

P. farinosus (Holm) Brown & Smith and *P. fumosoroseus* are important species which are used in the biological control of lepidopteran larvae. These fungi are used in the control of thrips and other insects like *Plutella xylostella*. *P. farinosus* is also called as yellow muscardine fungus.

***Paecilomyces farinosus* (Holm ex S.F. Gray) Brown & Smith**

- Colonies fast growing at first white turning bright yellow or cream, reverse cream or yellow

- Conidiphores smooth consisting verticillate branches with whorls of 2-4 phialides, 100-300X1.0-2.5 μ m
- Phialides swollen at base, tapering into distinct neck, 5-15X1.2-2.5 μ m in size
- Conidia ellipsoidal to fusiform, lemon shaped, smooth, hyaline, 2-3X1-2 μ m
- Chlamyospores absent

***Paecilomyces fumosoroseus* (Wize) Brown & Smith**

- Colonies slow growing, raised, floccose, pink, sometimes producing synnemata on agar medium
- Conidiphores erect bearing several compact whorls of phialides
- Phialides have strong inflated base
- Conidia cylindrical to fusiform, 3-4X1-2 μ m

Laboratory bioassay procedures for screening fungal pathogens on insects

Insect pathogens: *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii*, *Nomuraea rileyi*

Insects: *Helicoverpa armigera*, *Spodoptera litura*, *Plutella xylostella*, *Aphis craccivora*

Maintenance of fungi: The cultures are maintained in slants on PDA in refrigerator. For *N.rileyi*, Sabouraud maltose agar with yeast extract (SMAY) is used. The slants are subcultured once in every month. After 5-7 subcultures, the fungus is passed through their respective hosts.

Preparation of fungal inoculum for bioassays: The fungus is grown on PDA/SMAY medium for 7-10 days in slants and aqueous spore suspensions of various spore concentrations are prepared using sterile water. The spore count is estimated by Haemocytometer. (10^5 - 10^{10} spores/ml). Tween-80 is added @ 0.01% to get uniform spore suspension.

Rearing insects

<i>H.armigera</i> , <i>S.litura</i>	-	Artificial diet
<i>P.xylostella</i>	-	Mustard seedlings in cups/ trays
<i>A.craccivora</i>	-	Cowpea seedlings in cups/ trays

Stage of insect for bioassay

<i>H.armigera</i> , <i>S.litura</i> , <i>P.xylostella</i>	-	II, instar larvae
<i>A.craccivora</i>	-	Nymphs and adults

Method of inoculation

H. armigera

1. Spray the spore suspension on individual larva in petriplates with an atomizer or allow to crawl the larva on sporulated mat of the fungus in slants/ plates
2. Transfer the inoculated larva to fresh diet in vials (1 larva/vial)
3. Keep the vials in growth chambers at 25°C and 80-95%RH for 10 days

S. litura

1. Spray the spore suspension (10ml) on clean castor leaves both sides or alternately the spore suspension can be brushed with a fine brush.
2. Keep the sprayed leaves (5No.) in a vial containing water and the vial can be kept in a plastic container with a mesh lid
3. Release 10 larvae on five sprayed leaves and kept in growth chamber at 25°C and 80-95%RH for 10 days

P. xylostella

1. Spray the spore suspension on 70 young mustard seedlings grown in a cup/tray (20 ml/cup)
2. Release 15 larvae/cup and keep in growth chamber at 25°C and 80-95%RH for 10 days

A. craccivora

1. Rear *A. craccivora* on cowpea seedlings grown in small trays (5 seedlings/tray)
2. Spray the spore suspension on aphid infested cowpea seedlings grown in a cup/tray (5ml/tray)
3. Keep the sprayed plants in growth chamber at 25°C and 80-95%RH for 10 days

Control: Spraying with 0.01% Tween-80 solution

Observations at 24hr intervals

Mortality, cadavers with and without fungal growth, LC-50, LT-50.

Selected References

- Askew A J & Laing M D 1993 *Plant Pathology* 42 : 686-690.
Bisset J 1984 *Canadian Journal of Botany* 62:924-931.
Bisset J 1991 *Canadian Journal of Botany* 69: 2372-2417.

- Butt T M & Goettel M S 2000 **In:** Bioassays of entomogenous fungi. In: Bioassays of entomopathogenic Microbes and Nematodes. pp.141-191. CAB International.
- Dennis C & Webster J 1971 a *Transactions of British Mycological Society*, **57**: 25-39.
- Dennis C & Webster J 1971 b *Transactions of British Mycological Society*, **57**: 41-48.
- Dennis C & Webster J 1971 c *Transactions of British Mycological Society*, **57**: 363-369.
- Elad Y & Chet I 1983 *Phytoparasitica* **11**: 55-58.
- Elad Y, Chet I. & Henis Y 1981 *Phytoparasitica* **9**: 59-68.
- Gama W & Bissett J 1998 **In:** *Trichoderma and Gliocladium*. Volume-1. Basic biology and taxonomy. pp3-31. Tailor and Francis Ltd. London and USA.
- Gary A. Samuels 2004 *Trichoderma, a guide to identification and Biology*. USDA publication, Beltsville, MD20705, USA
- George O Poinar, Jr. & Gerard M. Thomas 1978 *Diagnostic Manual for the Identification of Insect Pathogens*. Plenum Press, New York and London. pp218.
- Ignoffo C M. 1988 *CRC Handbook of Natural Pesticides, vol.5. Microbial Insecticides, Part- A, Entomogenous Protozoa and Fungi*. CRC, Boca Raton, FL. pp. 151-236.
- Nagamani A, Manoharachari C, Agarwal D K & Chowdhry P N 2002 *Monograph contribution of Trichoderma*. pp 47. Associated Publishing Company, P.B No. 2679, Karol Bagh, New Delhi.
- Rifai, M A 1969 *Mycological Paper No.116*: pp 50.
- Samson RA, Evans HC & Paullatge J 1988 **In:** Atlas of Entomopathogenic Fungi, Springer - Verlag, Berlin, pp187.
- Tanada, Y. and Kaya, H.K. 1993. *Insect pathology*, Academic press Inc. California, USA.
- Webber J F. & Hedger J N 1986 *Transactions of British Mycological Society* **86** (1): 93-101.

Exploitation of the antagonistic microbes for identifying novel antifungal genes and plant disease management

R Viswanathan & P Malathi

Modern agriculture depends more on chemicals, which cause several environmental concerns, consumer health as well as the increasing incidence of fungicide resistance in pathogens. Moreover, the rapid evolution of new virulent forms of phytopathogens demands less expensive and time-consuming processes for genetic plant improvement. Hence, alternate strategies are to be developed to tackle plant diseases using eco-friendly approaches. Slowly biocontrol approach is getting acceptance in different developing countries as well as in non-traditional crops. Although developing disease resistance varieties through conventional approach would solve many disease problems, it is not feasible in many vegetatively propagated horticultural crops and in crops where breeding programme is lengthy e.g. sugarcane, coconut etc. Saprophytic microorganisms with potential to provide effective and useful biological control of plant pathogens already exist in the rhizosphere or phylloplane as part of the vast and largely untapped natural biological and genetic source. Biological control of pathogenic fungi may operate by antibiosis, competition and mycoparasitism. Mycoparasitism relies on the production of lytic enzymes by the mycoparasite for the degradation of cell walls of the host fungus. Antifungal and antibacterial proteins are key components of defense and offence mechanisms of many groups of fungi and bacteria. They are often effective on a broad range of targets and function synergistically in combinations, also with other biologically active compounds like antibiotics (Lorito *et al.* 1996). Many such genes have been identified, however only a few have been tested transgenically in plant to improve disease resistance. Chitinase encoding genes have been among the most used to improve plant defense against fungal pathogens.

Advancement in molecular techniques has enabled us not only to understand the molecular basis of disease resistance but also to produce many desired plant species. The development of plant transformation and gene cloning techniques has opened new avenues for augmenting disease and insect resistance in crops. In the past 25 years, the tremendous

achievements in technologies for identification, isolation and transfer of genes have enabled expression of traits for disease resistance in plants with minimal effects on their intrinsic properties. Since the first plant expressing a transgene was obtained by *Agrobacterium* mediated transformation, a variety of desirable traits have been transferred to model and crop plants by using a few but powerful techniques (Mourgues *et al.* 1998). The present lecture summarizes the role of microbial enzymes in biocontrol of plant pathogens, their characterization with special emphasis on chitinases and inactivation of phytotoxins produced by different plant pathogens by biocontrol organisms.

Microbial enzymes in biocontrol

Plants have evolved an array of defense systems to protect themselves against different biotic and abiotic stresses. One of these defense strategies includes the synthesis of a battery of proteins whose apparent function is to restrict the growth of an invading pathogen. These proteins include pathogenesis related (PR) proteins, such as chitinases and α -1,3-glucanases, which are expressed constitutively in tissues vulnerable to pathogen attack (Bishop *et al.* 2000). When an attack occurs, chitinases are highly expressed in and around the infected cells (Agrios 1997). Chitinases (EC 3.2.1.14), which are present in many plant species, hydrolyse the α -1,4-linkages of the N-acetylglucosamine (GlcNAc) homopolymer of chitin. Chitin along with α -1,3-glucan form the main skeletal polysaccharides of fungal cell walls (except those from Oomycetes, which contain α -glucans and cellulose) however, both are absent in plant cells (Cabib 1987; Gooday 1990). Chitinases are also found in a variety of other organisms, including most fungi, yeasts and some prokaryotes (Cohen 1993). Due to their hydrolytic ability, chitinases are able to inhibit the growth of fungal pathogens by inhibiting spore germination and germ-tube elongation, degrading the cell walls, as well as inhibiting growth at the hyphal tips (Chet & Inbar 1994, 1997; Haran *et al.* 1996). In addition to this direct action, chitinolytic breakdown products induce the production of defense compounds, such as phytoalexins, and systemic acquired resistance by acting as elicitors (Ren & West 1992; Bishop *et al.* 2000). It has been found that some plant chitinases may also display varying levels of lysozyme (EC 3.2.1.17) activity and thus may be involved in conferring resistance to bacterial pathogens (Jolles & Jolles 1984).

Chitinases and α -1,3-glucanases act as the key enzymes in the lysis of phytopathogenic fungal cell walls during the antagonistic action of

different microbes. However, other cell wall degrading enzymes (CWDEs), including those hydrolyzing minor polymers i.e., α -1,6-glucans or proteins may also be involved in the antagonistic response of *Trichoderma* (De la Cruz & Llobell 1999; Germia *et al.* 1993). Polysaccharides which consist of α -glucosidic linkages also appear to be important in the cell wall composition and architecture. The alkali-soluble glucose (S-glucans) range from polymers containing nearly pure α -1,3-linkages to polymers alternating α -1,3 and α -1,4- linkages. S-glucans represent the major matrix polysaccharides for most fungi (Benitez *et al.* 1998).

Many *Trichoderma* and *Gliocladium* spp. isolates obtained from natural habitats have been used in biocontrol trials against several soil borne plant pathogenic fungi under both green house and field conditions. The antagonists kill the host by direct hyphal contact, causing the affected cells to collapse or disintegrate; vegetative hyphae of all species have been found susceptible (Chernin & Chet 2002). Most of the studies on the expression and regulation of these lytic enzymes have been performed in liquid cultures supplemented with different C sources like chitin, glucose, α -1, 4-linked N-acetylglucosamine, fungal cell walls and their antifungal effects determined *in vitro*. A set of chitinolytic enzymes secreted by different antagonistic microbes, when grown on chitin as the sole C source consists N-acetylglucosaminidase, endochitinase and exochitinases. Chitinolytic enzymes of *T. harzianum*, the most studied mycoparasitic fungus, and no other chitinases have been reported with a broad range of activity, synergy between different enzymes, or synergy with a number of other biological and chemical control agents. Production of chitinases by *Trichoderma* spp. has been implicated as a main determinant in the suppression of fungal propagules (Lorito *et al.* 1993).

Roberts & Selitrennikoff (1988) studied plant and bacterial chitinases for anti-fungal activities and enzyme specificity. They reported that chitinases isolated from the plants functioned as endochitinases and from the bacteria function as exochitinases. Ordentlich *et al.* (1988) for the first time established that slower disease development by *Sclerotium rolfsii* on bean seeds in presence of *S. marcescens* to its high chitinase activity. Govindsamy *et al.* (1988) reported efficacy of purified chitinase from *M. verrucaria* to control *P. arachidis*. Later, Shapira *et al.* (1989) cloned *S. marcescens* chitinase gene and chitinase preparation obtained was found to be effective in reducing disease incidence caused by *S. rolfsii* in beans and *R. solani* in cotton under green house conditions. Vyas & Deshpande (1989) reported production of high levels of extracellular mycolytic enzymes viz. chitinase, α -1,3 glucanase and proteinase by *Myrothecium verrucaria* which are effective against *S. rolfsii* infecting groundnut. Several chitinolytic

bacteria, e.g. *Serratia marcescens*, *Aeromonas caviae*, *Enterobacter agglomerans*, *Bacillus* sp and *Pseudomonas fluorescens* have been reported to be effective antagonists for protecting plants against various fungal diseases (Inbar & Chet 1991; Chernin *et al.* 1995; El-Tarabily *et al.* 2000; Melentev *et al.* 2001; Neilsen *et al.* 1998). The chitinolytic rhizobacterium *Serratia plymuthica* isolated from the rhizosphere of oilseed rape was found to be an antagonist against *Verticillium dahliae*, *Rhizoctonia solani* and *Sclerotiana sclerotiorum*. Their chitinolytic enzymes inhibit spore germination, germ tube elongation and thus they are thought to be potential bioagents for the suppression of fungal propagules (Frankowski *et al.* 2001). A highly chitinolytic strain of *Alcaligenes xylosoxydans* utilized chitin as a source of carbon and nitrogen in the culture medium for growth and utilizes mycelia of *Rhizoctonia bataticola* and *Fusarium* sp. attacking pigeon pea, and the biocontrol activity of this strain was attributed to the production of chitinase (Vaidya *et al.* 2001). Certain cellulolytic microbes utilize chitin as a nitrogen source for degradation of cellulosic materials. For example *Cellulomonas uda* utilized chitin as a source of nitrogen for degrading cellulose thus, it has selective advantage over other cellulolytic microbes (Reguera & Leschine 2001). The antagonistic activity of antifungal bacterial strains against the grapevine dieback fungus, *Eutypa lata* was confirmed by the production of hydrolytic enzyme activity (Schmidt *et al.* 2001). Vasseur *et al.* (2001) screened several chitinase overproducing mutants of *Aphanocladium album* based on chitinolytic activity on agar medium containing colloidal chitin as carbon source. Sindhu & Dadarwal (2001) found that chitinase and cellulase produced by *Pseudomonas* strains showed mycelial growth inhibition of *Pythium aphanidermatum* and *R. solani* on PDA medium. Thus these studies indicate that in addition to *Trichoderma*, chitinases production by bacterial strains contribute to the inhibition of the pathogen. Similarly Folders *et al.* (2001) found that *P. aeruginosa* secretes many proteins into its extracellular medium and isolated the chiC gene encoding a polypeptide, which is able to bind and degrade colloidal chitin. Ajit *et al.* (2006) have studied the ability of fluorescent pseudomonads antagonistic to the vascular wilt of carnation *Fusarium oxysporum* f. sp. *dianthi* to produce chitinases on different substrates. Their studies established the significance of chitinases in the antagonism of fluorescent pseudomonads.

An early event in parasitism by *Trichoderma* spp is coiling of the *Trichoderma* spp around its host (pathogen). Later, the antagonist penetrates into the cells of the host, causing cell wall alterations, plasma membrane disorganization and aggregation of the cytoplasm. Benhamou & Chet (1993)

reported that damage to *R. solani* hyphae began soon after coiling of *T. harzianum* around its host, followed by the rapid collapse and loss of turgor of host hyphae, suggesting that lytic enzymes were produced by *Trichoderma* spp in response to contact between both the partners. A *Fusarium chlamydosporum* strain, a mycoparasite of groundnut rust, *Puccinia arachidis* produces an endo-chitinase that inhibits germination of uredospores of the rust fungus indicating a significant antifungal activity of chitinase (Mathivanan *et al.* 1998). Higher levels of antifungal activity were recorded when more chitinolytic/glucanolytic enzymes of *T. harzianum* are tested together. Combining a 42-kDa endochitinase and a 40-kDa chitobiosidase from *T. harzianum* strain P1 resulted in a synergistic increase in antifungal activity (Lorito *et al.* 1993; 1994). A variety of synergistic interactions have been found when different enzymes were combined or associated with biotic or abiotic antifungal agents (Chernin & Chet 2002).

Inglis & Kawchuk (2001) tested 14 fungi primarily representing mycoparasitic and biocontrol fungi for their ability to grow on and degrade cell walls (CWs) of an oomycete (*Pythium ultimum*), ascomycete (*Fusarium equisetii*), and basidiomycete (*Rhizoctonia solani*), and their hydrolytic enzymes were characterized. Protein was detected in the cultural medium of eleven of the test isolates, and these fungi significantly degraded CWs over the 14-day duration of the experiment. In general, a greater level of CW degradation occurred for *F. equisetii* and *P. ultimum* than for *R. solani*. Fungi that degraded *F. equisetii* CWs were *Coniothyrium minitans*, *Gliocladium roseum*, *Myrothecium verrucaria*, *Talaromyces flavus*, and *T. harzianum*. Taxa degrading *P. ultimum* CWs included *Chaetomium globosum*, *Coniothyrium minitans*, *M. verrucaria*, *Seimatosporium* sp., *Talaromyces flavus*, *T. hamatum*, *T. harzianum*, and *T. viride*. Production of extracellular protein was highly correlated with CW degradation.

Characterization of microbial enzymes

After establishing the role of microbial enzymes, characterization of them is vital to clone them in suitable vectors and exploiting them in biocontrol. The microbes are grown on specific substrates like purified pathogen cell wall or chitin and induced enzymes are purified and characterized by SDS-PAGE and other molecular techniques. Specific chromogenic substrates are used to identify different types of enzymes by colorimetric assay. Similarly specific substrates with fluorescent compounds are used in activity gel analyses to identify different enzymes with their activity. Extra cellular proteins synthesized by different microbes show

proteins of varying molecular weight in SDS PAGE. Earlier reports indicate that the molecular weight of microbial chitinases range from 20 kDa to 120 kDa (Fenice *et al.* 1998). The molecular weight of chitinase of *T. harzianum* Rifai T24 was reported as 43 kDa (El-Katatny *et al.* 2001). Inglis & Kawchuk *et al.* (2001) found a considerable variation in the molecular weights of CW-degrading enzymes among the test fungi and the CW substrates in zymogram electrophoresis. Multivariate analysis between CW degradation and hydrolysis of barley α -glucan (α -1,3- and α -1,4-glucanases), laminarin (α -1,3- and α -1,6-glucanases), carboxymethyl cellulose (endo- α -1,4- glucanases), colloidal chitin (chitinases), and chitosan (chitosanases) was conducted. For *F. equisetii* CWs, the regression model accounted for 80% of the variability, and carboxymethyl cellulases acting together with α -glucanases contributed an *Rf* of 0.52, whereas chitinases and α -glucanases alone contributed an *Rf* of 0.11 and 0.12, respectively. Only 61% of the variability observed in the degradation of *P. ultimum* CWs was explained by the enzyme classes tested, and primarily α -glucanases (*Rf* of 0.53) and carboxymethyl cellulases (*Rf* of 0.08) alone contributed to CW break down. Only few of the test fungi degraded *R. solani* CWs to perform multivariate analysis effectively. This study identified several fungi that degraded ascomyceteous and oomyceteous, and to a lesser extent, basidiomycetous CWs. Alexandre *et al.* (2002) have identified N-acetyl- α -D-glucosaminidase protein of 73-kDa and 68-kDa in two different truncated forms and endochitinase of 45-kDa. Also N-acetyl- α -D-glucosaminidase of 36-kDa has been purified from *T. harzianum* which controls *Crinipellis perniciosa* (Marco *et al.* 2004). The chitinase protein of Bbchit1 of 33-kDa was purified from *Beaveria bassiana* by Fang *et al.* (2005). Hoell *et al.* (2005) have isolated a novel endochitinase of 30-kDa from *T. atroviride*. Ait-Lahsen *et al.* (2001) purified and characterized one of the α -1,3-glucanases AGN13.1 from *T. harzianum*. The enzyme was exo type α -1,3-glucanase and showed lytic and antifungal activity against fungal plant pathogens *Aspergillus niger*, *Botrytis cinerea* and *Penicillium aurantiogriseum*. Extracellular proteins of highly antagonistic bacterial strain to carnation wilt pathogen were isolated from cell-free extracts of media amended with chitin and fungal cell wall. These cell-free conditioned media contained one to seven polypeptides. Western blot analysis revealed two isoforms of chitinase with molecular masses of 43 and 18.5-kDa (Ajit *et al.* 2006).

The mechanisms by which the chitinases are induced under mycoparasitic conditions are unknown. It has been established that a low-molecular weight, diffusible factor is released from the host and triggers chitinase gene expression in *T. atroviride* (Cortes *et al.* 1998; Zeilinger *et*

al. 1999). Kullnig *et al.* (2001) provided evidence that this factor is produced by an as yet unidentified chitinase of *Trichoderma*. Inbar & Chet (1995) using a biomimetic system, detected a N-acetyl- β -D-glucosaminidase as the first chitinase formed when *Trichoderma* comes into contact with its host.

Brunner *et al.* (2003) found that of nag1 of *T. atroviride* encodes a 73-kDa N-acetyl- β -D glucosaminidase which is secreted into the medium and partially bound to the cell wall and it has major impact on the induction of *T. atroviride* chitinase by chitin, its deletion virtually blocking the formation of other chitinases including *ech42*. This finding is of interest as it offers new insights into the mechanism of chitinase induction in *Trichoderma*. Table 1 summarizes most used chitinases identified from *Trichoderma* spp.

Table 1. Chitinase proteins purified from *Trichoderma* spp.

Species	Strain	Chitinase type	Mr (kDa)	References
<i>T. harzianum</i>	CECT2413	Endochitinase	42, 37, 33	De la Cruz <i>et al.</i> 1992
<i>T. atroviride</i>	P1	Endochitinase	42	Harman <i>et al.</i> 1992
		Chitobiosidase	40	
		N-acetyl- β -D-glucosaminidase	73	Lorito <i>et al.</i> 1994
<i>T. harzianum</i>	Nottingham39.1	Endochitinase	40,	Ulhoa & Peberdy, 1992
		N-acetyl- β -D-glucosaminidase	64	Ulhoa & Peberdy, 1992
<i>T. harzianum</i>	Nottingham39.1	Endochitinase	46	Lima <i>et al.</i> 1997
<i>T. harzianum</i>	Nottingham39.1	Exochitinase	28	Deane <i>et al.</i> 1998
<i>T. harzianum</i>	AF6-T8	N-acetyl- β -D-glucosaminidase	46	Lima <i>et al.</i> 1997

Cloning of genes coding for microbial enzymes

Cloning of genes encoding for lytic enzymes, characterizing their products, and elucidating their individual roles in the mycoparasitic activity of *T. harzianum* and other species of *Trichoderma* or *Gliocladium* has opened the way to improve the biocontrol capacity of these fungi. Also the genes coding for these lytic enzymes were expressed in plants to resist invading pathogens. Several genes which are specifically induced under mycoparasitic conditions have been cloned from *Trichoderma* spp, chitinase-encoding genes are prominent among these. Three endochitinases (*ech 42*, *chit 33*, *chit 36*) and two chitinases (*nag1 = exc1*, *exc2*) have been cloned and characterized (Lorito *et al.* 1998; Kubicek *et al.* 2001; Viterbo *et al.* 2001; Kim *et al.* 2002). Cloning of genes encoding for endochitinase

and N-acetylglucosaminidase from *Trichoderma* spp. was reported by various workers (Harman *et al.* 1993; Haran *et al.* 1995; Carsolio *et al.* 1994; Giczey *et al.* 1998; Limon *et al.* 1999; Peterbauer *et al.* 1996). The chitinase gene ech42 of 1447bp long was obtained from *T. atroviride*, which had three introns in the sequence (Jin Zhu *et al.* 2005). A list of important chitinase genes cloned from different *Trichoderma* spp. is given in Table 2.

Table 2. Chitinase genes cloned from *Trichoderma* spp.

Gene	<i>Trichoderma</i> spp.	Strain	Encoded protein	References
<i>Th-En42</i>	<i>T. atroviride</i>	P1	42-kDa Endochitinase	Hayes <i>et al.</i> 1994
<i>ech42</i>	<i>T. atroviride</i>	IMI 206040	42-kDa Endochitinase	Carsolio <i>et al.</i> 1994
<i>chit42</i>	<i>T. harzianum</i>	CECT2413	42-kDa Endochitinase	Garcia <i>et al.</i> 1994
<i>chit4</i>	<i>T. virens</i>	Gv29-8	42-kDa Endochitinase	Beak <i>et al.</i> 1994
<i>th-ch</i>	<i>T. hamatum</i>	Tam-61	42-kDa Endochitinase	Fekete <i>et al.</i> 1994
<i>ENC1</i>	<i>T. harzianum</i>	T25-1	42-kDa Endochitinase	Draborg <i>et al.</i> 1994
<i>Chit33</i>	<i>T. harzianum</i>	CECT2413	33-kDa Endochitinase	Limon <i>et al.</i> 1994
<i>Nag 1</i>	<i>T. atroviride</i>	P1	73-kDa N-acetyl- β -D-glucosaminidase	Peterbauer <i>et al.</i> 1994
<i>exc 1</i>	<i>T. harzianum</i>	T25-1	73-kDa N-acetyl- β -D-glucosaminidase	Draborg <i>et al.</i> 1994
<i>exc 2</i>	<i>T. harzianum</i>	T25-1	N-acetyl- β -D-glucosaminidase?	Draborg <i>et al.</i> 1994

Cloning microbial chitinase from antagonists to sugarcane red rot pathogen

We are working for the past ten years on biocontrol and induced systemic resistance using fluorescent pseudomonads and *Trichoderma* spp against *Colletotrichum falcatum* causing red rot of sugarcane (Viswanathan 2006). Studies of Viswanathan & Samiyappan (2000) established that strains of fluorescent pseudomonads suppress red rot development in sick-plot conditions. Subsequently induced systemic resistance against the pathogen under field conditions has also been demonstrated (Viswanathan and Samiyappan 2002). In addition to the work on disease management detailed studies were conducted on identifying and characterizing extracellular enzymes produced by the antagonists and cloning of chitinases from strains of *T. harzianum* and *Pseudomonas fluorescens* native to sugarcane

Initially we have standardized conditions favourable for the synthesis of chitinases by *Pseudomonas* strains in different culture media and established correlation between chitinase production by the bacterial strains and their antagonism to *C. falcatum* (Viswanathan & Samiyappan 2001). In the further studies efficient chitinolytic *Pseudomonas* and *T. harzianum* strains were selected based on their ability to degrade colloidal chitin on

chitin agar medium. Growing bacterial strains using colloidal chitin as a carbon source in the agar medium and visualizing the clearing zone around the antagonist colony was found to be an easy and simple method of screening for chitinolytic strains. Hydrolytic enzyme production by the bacterial strains was compared with that of *T. harzianum* strains. Among 24 different bacterial strains screened for the chitinolytic activity *Pseudomonas* strains AFG2 and FP7 were highly efficient in chitin degradation along with *T. harzianum* strains T5 and T62. The extracellular proteins from the efficient strains caused inhibition to *C. falcatum* conidial germination, germ tube elongation and mycelial growth. We also found that enzyme extracts of *T. harzianum* are more antifungal to *C. falcatum* than those of the bacterial antagonists (Viswanathan *et al.* 2003a).

Profiles of extracellular proteins produced by *Pseudomonas* AFG4 on *C. falcatum* cell wall and chitin medium showed proteins in the range of 20 to 97-kDa. Colorimetric assay using chromogenic *p*-nitrophenyl substrates for the production of different chitinases revealed that *Pseudomonas* AFG4 produced more chitinolytic enzymes than *T. harzianum* T5. It was found that both the strains produced more enzymes in cell wall medium, and N-acetyl- β -D-glucosaminidase and chitin-1,4- β -D-N,N'-chitobiosidase were produced in higher quantities than chitin- β -D-N,N',N''-chitotriase. Even though *Pseudomonas* AFG4 showed chitinolytic activity in the colorimetric assay, we could not detect any fluorescent band in the activity gel for chitobiase and chitotriase as in *T. harzianum* (Viswanathan *et al.* 2003b).

Further characterization of *T. harzianum* extracellular chitinases was carried out by SDS-PAGE, which showed extracellular proteins in the range of 20 to 124-kDa. We have standardized detection of chitinase isoforms in SDS-PAGE gels after reactivation by casein- EDTA procedure. In this procedure, activity of chitinase isoforms was detected as discrete fluorescent bands under UV light which after few moments diffused. The fungus produced N-acetyl glucosaminidase of 97-kDa both in cell wall and colloidal chitin medium, which was detected in the overlay gel using 4-MU- (GlcNAc) as substrate. Three fluorescent bands with approximate molecular weights of 66-kDa, 56-kDa and 50-kDa were detected when grown in cell wall amended medium and two bands of 66-kDa and 50-kDa in colloidal chitin amended medium using 4-MU- (GlcNAc)₂ as substrate. Also two fluorescent bands of 66-kDa and 50-kDa in cell wall medium and a 50-kDa band in colloidal chitin medium using 4-MU- (GlcNAc)₃ as substrate were detected. These fluorescent bands on the basis of their

molecular weights overlap to the stained proteins in the SDS-PAGE (Viswanathan *et al.* 2004).

In our further studies efforts were made to isolate the chitinase gene(s) from *P. fluorescens* AFG4 and *T. harzianum* T5 for which procedures were standardized to isolate total RNA and mRNA, RT-PCR, cDNA cloning and transformation of recombinant plasmids in competent cells. The cDNA products were sequenced and using GenBank BLAST programme both the sequences were compared. The sequencing results gave the partial cDNA products of 246bp and 230bp by *T. harzianum* T5 and *P. fluorescens* AFG4 respectively. The homology analysis of the partial cDNA sequences with the existing sequences in GenBank revealed a very high level of identity at the nucleotide level and protein level to the reported microbial chitinases (Viswanathan *et al.* 2004; 2006). This partial cDNA sequence of chitinase will be used as probe to pick the full-length chitinase gene later.

Developing transgenic plants with microbial genes

Antifungal and antibacterial proteins are key components of defense and offence mechanisms of many groups of fungi and bacteria. They are often effective on a broad range of targets and function synergistically in combinations, also with other biologically active compounds like antibiotics (Lorito *et al.* 1996). Although a number of such genes have been identified, not many have been tested transgenically in plant to improve disease resistance. Chitinase encoding genes have been among the most used to improve plant defense against fungal pathogens. The antifungal activities of chitinases render these proteins ideal candidates for the enhanced natural resistance of plants through constitutive over expression of these normally inducible defense genes. Successes in this regard were first reported by Broglie *et al.* (1991) who generated transgenic tobacco and canola plants utilizing the cauliflower mosaic virus (CaMV) 35S promoter and the bean *CH5B* gene. Chitinase activity in the transformants increased 44-fold in the leaves, relative to the control plants. The transformants showed delayed development of disease symptoms, as well as an improved ability to survive in soil infected with *R. solani*. The first attempt to use a transgenic chitinase to enhance plant defense were made by using the bacterial chitinase gene *ChiA* from *Serratia marcescens*. Jones *et al.* (1988) demonstrated the feasibility of accumulating the *S. marcescens* chitinase in tobacco leaves under two different promoters, while Suslow *et al.* (1988) evaluated the role of transformed chitinase in plant defense by using the same gene. They established that expression of a bacterial chitinase gene *ChiA* from

S. marcescens in tobacco plants with CaMV 35S promoter confers increased resistance to *Alternaria longipes* by reducing necrotic lesions and chlorosis during infection. However, the increase in resistance diminishes as the plant ages (Suslow *et al.* 1988) and exhibited tolerance to *R. solani* infection in the field (Howie *et al.* 1994). Terakawa *et al.* (1997) transferred *Rhizopus oligosporus* chitinase gene (*chi1*) involved in cell autolysis in tobacco. The transgenic plants showed 3 to 4 fold increase in chitinase activity and they detected a significant reduction in leaf symptoms caused by *S. sclerotiorum* and *B. cinerea*.

T. harzianum *ech42* gene encodes one of the most powerful chitinolytic enzymes characterized so far, which is capable of degrading rapidly any chitin containing fungal structures like chlamydospores, mature hyphae and sclerotia and inhibits a broad range of fungi *in vitro* (Lorito & Scala 1999). The fungal gene was expressed constitutively at high level in plant and transgenic lines upto 400 fold more chitinolytic than controls were obtained with no apparent effects on plant development. The transgenic expression of the *ech42* gene in tobacco and potato conferred almost complete resistance to the aerial pathogens *Alternaria alternata*, *R. solani*, *B. cinerea* and to the soil borne pathogens *R. solani* and *S. sclerotiarum* (Lorito *et al.* 1998). The transgenic plants, in addition to the direct antifungal effects of the transgenic chitinase, other mechanisms of the plant defense system may be activated and help reaching a high level of resistance. Their studies conclusively demonstrated the usefulness of the fungal genome as a source of disease resistance genes, which apparently overcome limitations of using plant chitinase genes. Following this approach, *T. harzianum* genes have been transferred into a variety of crops, including, apple, petunia, pea, chickpea, rice, brassica, wheat, grape, alfalfa, tomato etc. and positive results have been obtained with apple against *Venturia inaequalis*, grape against *Uncinula necatar* and petunia against *R. solani* (Bolar *et al.* 2000, Esposito *et al.* 1999). However, in apple, the expression of even moderate levels of enzyme caused a reduction in plant vigour (Bolar *et al.* 2000) even though no such effects were seen in tobacco or potato (Lorito *et al.* 1998).

Use of a single gene for developing transgenic plant could have several potential drawbacks. First, any single disease-control gene that is used widely is likely to encourage the development of resistance to the effective molecule in the pathogen population as a consequence of selection (Wenzel 1985; Jach *et al.* 1995; Fuehs 1998). Second, the use of single gene may require higher levels of heterologous protein expression for

adequate levels of resistance than the use of two or more genes that encode synergistic proteins. Lower production of total heterologous proteins would reduce the diversion of plant resources into production of these proteins. Act of these goals could be met with additional genes for disease resistance, especially if the proteins produced by them had strong synergy in their antifungal activity. All of these goals could be met with additional genes for disease resistance, especially if the proteins produced by them had strong synergy in their antifungal activity.

An exochitinase, N-acetyl- β -D-hexosaminidase (nag70, EC 3.2.1.52), and the endochitinase from the biocontrol fungus *T. atroviride* are each strongly antifungal (ED50 values of about 50 μ g/ml for inhibition of conidial germination of *B. cinerea*) and strongly synergistic (ED50 values of 9 μ g/ml of total protein for a 1:1 mixture of the two proteins) (Lorito *et al.* 1994a, b). Wong *et al.* (1999) demonstrated *in vitro* synergism of *T. atroviride* exochitinase and endochitinase against *V. inaequalis*, the causal agent of apple scab and *Gymnosporangium juniperi-virginianae*, the causal agent of cedar apple rust. They are therefore good candidates for conjoint transfer into apple. The genes encoding both enzymes have been cloned from *T. atroviride* strain P1 and both contain a signal peptide at the 5' end that probably is responsible for extracellular secretion of the enzyme (Hayes *et al.* 1994; Draborg *et al.* 1995; Peterbauer *et al.* 1996). Bolar *et al.* (2001) have successfully transferred these two genes encoding for chitinolytic enzymes from *T. atroviride* in combination into apple plant. They found transgenics produced from cDNA version of exochitinase had a higher level of enzyme expression than lines produced from the genomic version. Constitutive expression of two fungal chitinases in apple lines was correlated with increased resistance to *V. inaequalis*. Low expression of endochitinase with low expression of exochitinase resulted in high levels of resistance with minimal effects on plant growth. However, lines with high levels of exochitinase in combination with very low levels of endochitinase had adequate plant vigour and very high levels of diseases resistance. Work of this group is a milestone in transgenic approach to manage plant diseases using microbial genes. This is the first report to demonstrate synergism by two chitinases of fungal origin in plants. Carstens *et al.* (2003) evaluated this CTS 1-2 gene from *Saccharomyces cerevisiae* for its *in planta* antifungal activity by constitutive over expression in tobacco plants and found that leaf extracts from the transgenic line inhibited *B. cinerea* spore germination and hyphal growth by upto 70% in a quantitative *in vitro* assay, leading to severe physical damage on the hyphae. On detached leaf infection assay, they found a decrease in susceptibility ranging from

50 to 70% to *B. cinerea*. The transgenic plant lines that showed increased disease tolerance were also shown to have higher chitinase activities.

The apple scab is routinely controlled in commercial apple orchards by multiple applications of chemical fungicides. Although great improvements have been made in the efficacy and safety of modern fungicides, they remain an economic burden to growers, and there is continuing concern for their non-target effects (Gadoury *et al.* 1989). 'McIntosh' is by far the most widely-planted apple cultivar in the northeastern United States, and like most commercial cultivars, is very susceptible to scab (Merwin *et al.* 1994). Conventionally bred scab resistant cultivars, such as 'Liberty' (Lamb *et al.* 1979) have inadequate quality for national and international markets. However by the transgenic approach 'McIntosh' variety expressing chitinases a stable resistance to apple scab was achieved (Bolar *et al.* 2001).

Microbial inactivation of pathogenicity determinants

Detoxification

A disease is said to be set in when a compatible interaction between plant pathogen and its host is established. In such a compatible interaction the plant is rendered susceptible to the invading pathogen. Any relationship between pathogen and its host in reality involves interactive molecular processes comprising plant's genes controlling resistance/susceptibility on the one hand and avirulence/virulence genes of the pathogen on the other (De la Fuente-Martinez & Herrera Estrella 1993). Since a compatible interaction is established, the nature of subsequent biological impairments caused by the pathogen largely depends on a variety of biochemical blueprints encompassing production of lytic enzymes and/or toxins by the pathogen (Panopoulous and Peet, 1985). Enzymes secreted by pathogen cause disruption of plant cell wall barrier while making the protoplasm vulnerable to attack by toxins which tear apart the naked protoplasm with an eventual loss of electrolytes and ultimate death of cell (Vidyasekaran 1997).

Detoxification of phytotoxins

Detoxification or inactivation of the phytotoxin reduces the toxicity of metabolite produced by plant pathogens. This process leads to development of resistant reaction or act as a defense mechanism in susceptible plants to protect them from pathogen infection. Toxin resistant genes can be isolated from the pathogen itself, as well as from other

microbes. Microorganisms form an exotic source of enzymes, which are capable of inactivating synthetic chemicals that are potentially phytotoxic (De Block *et al.* 1989). Studies are now being carried out on transformation of plants utilizing phytotoxin resistance genes of microbial origin. In safflower, *Alternaria carthamii* causing leaf and stem blight produces a non specific phytotoxic brefeldin A (BFA) which could be detoxified by a strain of *Bacillus subtilis* BG3. The bacterial strain was able to survive on BFA by simply converting it to a nontoxic metabolite by means of a brefeldin A esterase (BFA esterase) in a single step hydrolysis reaction. The gene was found to encode a 40kDa monomeric peptide. The esterase gene was subcloned and expressed in *Escherichia coli*. A fusion protein so obtained was found to have esterase activity three times that of the wild type esterase purified from *B. subtilis* brefeldin A. Esterase gene holds much promise in engineering blight resistance in safflower (Kneusel *et al.* 1994). Detailed studies were conducted by Malathi *et al.* (2002) on the possible detoxification of phytotoxin produced by the sugarcane red rot pathogen *C. falcatum* by antagonistic fungal and bacterial strains. Eleven *P. fluorescens* strains and two *T. harzianum* strains were grown on a medium containing the pathogen toxin. Later, the treated toxin was tested for its phytotoxic activity using symptom bioassay, electrolytic leakage and spectral analysis. In symptom bioassay, spot application of untreated toxin on leaf segments produced brown, necrotic spots with a yellowish-brown margin and a yellow halo, which elongated along the veins within 48-72 h. The phytotoxin incubated with *P. fluorescens* strains FP7 and VPT4 and *T. harzianum* strain T5 did not exhibit any symptom on susceptible sugarcane leaves. Electrolyte leakage studies revealed that there is an overall reduction in loss of electrolytes caused by the growth of antagonists in toxin medium. Also, spectral analysis showed varied spectral patterns by different treatments. The results revealed that certain strains of the biocontrol agents cause detoxification of the pathogen toxin, which is one of the major pathogenicity determinants of the red-rot pathogen. At molecular level a specific induction of extracellular high molecular protein (97kDa) from antagonistic strains has been identified through SDS-PAGE and it is under purification and characterization. Detoxification of fusaric acid (FA) using different microorganisms including fungal and bacterial isolates has been reported previously (Toyoda *et al.* 1991, Utisumi *et al.* 1988, 1991). The FA-detoxifying genes from the air-borne fungal isolate *Cladosporium weraeckii* and FA-resistance gene from *Pseudomonas cepacia* were cloned and sequenced (Utisumi *et al.* 1988; 1991). Thangavelu *et al.* (2001) conducted detailed studies on detoxification of FA of *Fusarium oxysporum*

f. sp. *cubense* by different antagonistic strains. They found an antagonist *P. fluorescens* Pf10 grows well in the FA amended medium and detoxified it completely under *in vitro* conditions. The detoxification of FA by *P. fluorescens* was demonstrated in tomato by the loss of wilt inducing ability of the treated toxin. Growing *P. fluorescens* in toxin-amended medium induced a 60kDa protein predominantly. The N-terminal amino acid sequence of this protein showed homology with amino peptidase of *P. aeruginosa*. Presence of gene coding for detoxification in plasmid has been confirmed by plasmid curing. Under greenhouse conditions, soil application of Pf10 reduced the wilt incidence by 50% whereas the plasmid deficient strain (Pf10P) failed to control the *Fusarium* wilt.

R. solani toxin (Rs-toxin) produced by the sheath blight pathogen *R. solani* induces sheath blight symptoms that are indistinguishable from those induced by the fungus. This toxin is a carbohydrate compound containing mainly α -glucose and mannose (Vidhyasekharan *et al.* 1997; Sriram *et al.* 2000). Studies of Sriram *et al.* (2000) revealed that certain isolates of *T. viride* were found to readily inactivate the toxin. *T. viride* MNT7 reduced severity of toxin-induced symptoms besides bringing about a sharp fall in electrolyte from toxin treated rice plant. They also found that a 110-kDa extracellular protein from *T. viride* restrict Rs-toxin activity to a significant extent. In the subsequent studies Shanmugam *et al.* (2001) confirmed that the inactivating principle of *T. viride* MNT7 was an α -glucosidase, a 110-kDa protein with a K_m value of 0.40 mmol/l, when p-nitrophenyl α -D-glucopyranosidase was the substrate, and an isoelectric point of 5.2 and it was highly specific to Rs-toxin. Fakhouri *et al.* (2003) reported detoxification of FA by a non-pathogenic *Colletotrichum* sp. Their studies revealed that the fungus isolate is able to completely detoxify after 4 days of incubation in malt broth medium containing 200 ppm of FA into 4-butyl-2-carboxy-pyrimidine. The detoxified compound did not have any toxic effect on tomato seedlings.

Leaf scald is one of the most devastating diseases of sugarcane in many countries, and the causal agent, *Xanthomonas albilineans*, is a systemic, xylem-invading bacterium that may be present at low population sizes in symptom-less plants. The pathogen produces a family of antibiotics and phytotoxins that block DNA replication in bacteria and sugarcane proplastids (Bireh & Patil 1985; 1987). The major toxin, named albicidin has been partially characterized as a low *Mr* compound with several aromatic rings. Besides albicidin is rapidly bactericidal to a range of Gram-positive and Gram-negative bacteria at concentrations as low as 1 ng ml⁻¹. Symptom

of leaf scald disease include the emergence of chlorotic leaves, wilting, necrosis, and sometimes rapid death of plants, often after a prolonged latent period. Chlorosis results from blocked chloroplast development after inhibition of plastid DNA replication by albicidins produced by the pathogen in invaded xylem (Birch & Patil 1987). Albicidin phytotoxins appear to be important in systemic disease development, because tox^- mutants of *X. albilineans* fail to induce any disease symptoms in inoculated sugarcane (Birch & Patil 1987). However, rapid attenuation of the pathogen in culture has prevented critical experiments comparing the capacity of tox^- mutants and tox^+ revertants to induce systemic disease in the field. Albicidin resistance genes are therefore of great interest as a critical tool to assess the role of albicidin phytotoxins in leaf scald development and potentially to confer leaf scald resistance in transgenic sugarcane. Several mechanisms of albicidin resistance have been identified, by production of a protein that reversibly binds the antibiotic in *Klebsiella oxytoca* (Walker *et al.* 1988) and in *Alcaligenes denitrificans* (Basnayake & Birch 1995) and irreversible detoxification of albicidin in *Pantoea dispersa* (Zhang & Birch 1997). A gene (*AlbD*) from *Pantoea dispersa* has been cloned and sequenced and been shown to code for a peptide of 235 amino acids that detoxifies albicidin. The gene shows no significant homology at the DNA or protein level to any known sequence, but the gene product contains a GxSxG motif that is conserved in serine hydrolases. The *AlbD* protein, purified to homogeneity by means of a glutathione *S*-transferase gene fusion system, showed strong esterase activity on *p*-nitrophenyl butyrate and released hydrophilic products during detoxification of albicidins and *AlbD* is an albicidin hydrolase. The enzyme detoxifies albicidins efficiently over a pH range from 5.8 to 8.0, with a broad temperature optimum from 15 to 35°C. Expression of *AlbD* in transformed *X. albilineans* strains abolished the capacity to release albicidin toxins and to incite disease symptoms in sugarcane. The gene is a promising candidate for transfer into sugarcane to confer disease resistance.

Trichoderma on pathogenic enzymes

The cell wall is the first barrier that plant cell uses to oppose attack of pathogens. Synthesis of hydrolytic enzymes by fungal pathogens during the first phase of the host pathogen interaction is crucial for the infection process. Most phytopathogenic microorganisms produce enzymes that are capable of degrading cell wall polymers. These enzymes include cutinase, which hydrolyses ester linkages of the cutin polymer and cell wall degradation enzymes i.e. pectolytic enzymes [exo- and endo polygalacturonases (PG), pectate lyase (PL), pectin methyl esterase (PME)]

and cellulolytic (Cx) enzymes. Most studied is the polygalacturonases, which cleaves the linkage between D-galacturonic acid residues. Attempts were made to study the interference of *Trichoderma* and other antagonist in hydrolytic enzyme production by the pathogens. Interference with pathogenicity processes by *Trichoderma* has been demonstrated in *T. harzianum* T39 and *B. cinerea* on bean leaves (Zimand *et al.* 1996). Here, the mechanism of biocontrol by which the antagonist interferes with the pathogenicity process indicates that either it could be in direct action of T39 on *B. cinerea* hydrolytic enzymes or it could be an indirect effect by affecting plant factors that induce enzymatic activity in *B. cinerea*. Further studies of Kapat *et al.* (1998) revealed that the isolate T39 inhibits germination of *B. cinerea* conidia and subsequent disease development. The levels of various hydrolytic enzymes were reduced when *B. cinerea* was grown with a *T. harzianum* isolate in liquid culture or on the surface of bean leaves. Significant reduction in production of pectin esterase, exo-PG, endo-PG, PME and PL in both systems was found. *T. harzianum* polygalacturanase inhibiting proteins in defense against phytopathogenic fungi was also reported (Lorenzo & Ferrari 2002). In addition protease enzyme is involved in the degradation of pathogen cell walls, membranes and even proteins released by the lysis of the pathogen, thus making nutrients available for the mycoparasite (Goldman *et al.* 1994). Elad & Kapat (1999) studied in detail on the role of *T. harzianum* protease in the biocontrol of *B. cinerea* *in vitro* and *in vivo* on bean leaves. They found that in the presence of *T. harzianum* T 39 culture filtrate containing protease on 55% reduction in the germ tube length 17 hr of incubation on the surface of the leaves, the protease reduced 56-100% reduction on *Trichoderma* protease of *B. cinerea* on the surface of bean leaves and the development of disease could be bimodal. First, protease may have an adverse effect on germination of *B. cinerea* conidia and secondly it may be able to deactivate the pathogen hydrolytic enzymes that are responsible for plant tissue necrosis.

Synergism of toxins and cell wall degrading enzymes

Previous work on *Trichoderma* metabolites has shown that CWDEs and membrane-acting peptides (i.e. trichorizianines and other peptaibols) concurrently produced during biological control interact synergistically as biocontrol agents (Lorito *et al.* 1996). Recent research on necrosis-inducing phytopathogenic pseudomonads had led to the isolation and structural characterization of two different groups of cyclin lipodepsipeptides (LDPs) that are toxic to plants and microorganisms. Fogliano *et al.* (2002) found *Pseudomonas syringae* pv. *syringae* strain B259 secretes two LDPs

syringomycin E (SRE) and syringopeptin (SP_{25A}) together with at least four types of cell wall degrading enzymes (CWDEs). In antifungal bioassays, they established the purified toxins interacted synergistically with chitinolytic and glucanolytic enzymes purified from the same bacterial strain or from the biocontrol fungus *T. atroviride* strain PI. The synergism between LDPs and CWDEs occurred against all seven different fungal species viz. *Fusarium oxysporum*, *Verticillium dahliae*, *B. cinerea*, *Penicillium expansum* *Phytophthora infestans*, *Rhodotorula pilimanae*, *T. atroviride* and *P. syringae* pv *syringae* itself, with a level dependent on the enzyme used to permeabilize the microbial cell wall. The antifungal activity of SP_{25A} was much more increased by the CWDE action than was that of the smaller SRE, suggesting a stronger antifungal role for SP_{25A}. *In vivo* biocontrol assays using *P. syringae* alone or in combination with *T. atroviride*, including a *Trichoderma* endochitinase knock-out mutant in place of the wild type and a chitinase-specific enzyme inhibitor they found synergistic interaction LDPs-CWDEs is involved in the antagonistic mechanism of *P. syringae* and they support the concept that a more effective disease control is given by the combined action of the two agents. Their results clearly demonstrated that enzymatic degradation of the cell wall permits both type of toxins, especially those with higher molecular weight (SPs), to reach their target and alter cell membrane functions much more effectively than in the absence of the enzymes. Malathi (Unpublished) has established a correlation between disease expression and production of secondary metabolites viz., toxin and enzymes viz. cellulolytic (cellulase) and pectinolytic (exo and endo PGs, PME) enzymes by the pathogen *C. falcatum*. She further established that reduction in symptom production by the pathogen on sugarcane due to microbial inactivation of enzymes. Studies are in progress to characterize the protein(s) at gene level.

Future prospects and conclusions

Microbes can provide useful genes that are either absent in plants or more potent in biological activity than their plant equivalent. A typical example, which involves compounds with direct antimicrobial activity, considers the use of antifungal chitinolytic enzymes. Given the proven abilities of agriculturally useful *Trichoderma* strains to secrete enzymes and other antimicrobial compounds, a lot of attention has been focused on the role of CWDEs and antibiotics in mycoparasitism or antagonism with phytopathogenic microbes (Kubicek *et al.* 2001). Although studies based on mutants and targeted gene disruption have been carried out by different groups, the redundancy in the *Trichoderma* genome of CWDE-encoding

genes has made it difficult to identify enzymes required for biocontrol independently of the strain and system used. The progress made in this area of work clearly suggests that these fungi have a variety of molecular weapons that can be used in different combinations depending on the microbe they are confronting or the plant they are colonizing. However, this has also demonstrated the abundance of biotechnologically valuable proteins and secondary metabolites that *Trichoderma* can provide and much less has been done on identifying genes and proteins that play a role in the interaction between the different *Trichoderma* strains and the plant. The complexity of the system, which requires more than one player to act at the same time has strongly indicated that a more holistic approach is necessary to make a leap forward in our understanding of the biological processes involved. For these reasons, several groups have recently started using proteomics (Grinyer *et al.* 2005) and functional genomics analysis in the attempt to obtain an overall picture of the changes that occur in the *Trichoderma*, plant, pathogen expressomes when they “talk” to each other, especially when an increase in disease resistance is generated (Woo *et al.* 2006).

Trichoderma expressed sequence tag (EST) libraries made under a large variety of inducing conditions are being processed, and entire genome sequencing initiatives have been launched both in United States and Europe. The studies performed on the proteome of *Trichoderma* spp. interacting with pathogens and plants have already provided many novel data that improve our understanding on how these fungi search for pathogen, talk to the plant, and protect themselves from toxicants. *Trichoderma* sp. is able to produce at least three different types of elicitors made of enzymes or peptides, *avr* proteins and oligosaccharides or low molecular weight compounds released by the action of specific *Trichoderma* enzymes on fungal and plant cell walls (Woo *et al.* 2006).

Understanding the mechanism of interaction between *Trichoderma* spp. and the plant has provided for the first time that the opportunity to genetically increase the ability of a *T. atroviride* strain to induce systemic resistance. Brunner *et al.* (2005) using *Trichoderma* strain P1 over expressing a glucose oxidase gene from *Aspergillus niger* established that the mutants perform much better than wild type both as mycoparasite and ISR inducing agents. In this case, the mutant spores applied to bean seeds produced plants which were more resistant to leaf infections by *B. cinerea* compared with those treated with wild type, mainly because of the high glucose oxidase activity expressed by the genetically improved *Trichoderma*

spp. catalyzed the production of hydrogen peroxide and reactive oxygen species that systemically altered the plant defense mechanism. Proteome and genome analysis have greatly enhanced our ability to conduct holistic and genome-based functional studies. Woo *et al.* (2006) have identified and determined the role of a variety of novel genes and gene-products, including ABC transporters, enzymes and other proteins that produce or act as novel elicitors of induced resistance, proteins responsible for a gene-for-gene avirulent interaction between *Trichoderma* spp. and plants, mycoparasitism-related inducers, plant proteins specifically induced by *Trichoderma*, etc.

However, an approach based on proteomics alone may not be sufficient to obtain a complete data set for describing microbial interactions. Therefore, further studies are necessary for proteins whose quantitative profile changes, e.g. by generating knockout strains for phenotypic analysis. Despite some inherent limitations, proteomics is a useful method, and an important complement to other approaches for studies of microbial interactions and identifying novel antifungal genes.

The work performed so far with microbial genes indicates that a significant improvement of plant resistance may be obtained in a variety of ways, but attained mainly by producing enzymes that degrade pathogen structures, enzymes that synthesize antimicrobial compounds or selective toxins. However each approach needs a tailor made strategy to minimize any detrimental effects on the acceptor plant and the expression system. A responsible use of fungal and bacterial genes for genetic improvement should produce a minimal effect on the ecosystem and contribute safely to a more productive and less chemically dependent agriculture. Work in the country on cloning and identifying new antifungal genes from microbes is scanty and more studies are required by different groups to cater to our need.

Selected references

- Agrios G N 1997 How plants defend themselves against pathogens. In: *Plant Pathology* (4ed.) (pp. 93-114) Academic Press, California.
- Ait-Lahsen H, Soler A, Ray M, De la Cruz J, Monte E & Llobell A 2001 *Appl. Environ. Microbiol.* 67: 5833-5869.
- Ajit N S, Verma R & Shanmugam V 2006 *Curr. Microbiol.* 52: 310-316.
- Alexandre A P F, Cirano J U, Marcelo V S, Edivaldo X F F & Carlos A O R 2002 *Brazilian J. Microbiol.* 33: 169-173.

- Basnayake W V & Birch R G 1995 *Microbiol.* 141: 551-560.
- Benhamou N & Chet I 1993 *Phytopathology* 83: 1062-1071.
- Benitez T, Limón C, Delgado-Jarana J & Rey M 1998 In: *Trichoderma and Gliocladium: Enzymes, Biological Control and Commercial Applications*, Vol.2. Taylor and Francis, Ltd, London, United Kingdom.
- Birch R G & Patil S S 1985 *J. Gen. Microbiol.* 131: 1069-1075.
- Birch R G & Patil S S 1987 *Physiol. Mol. Plant Pathol.* 30: 207-214.
- Bishop J G Dean A M & Mitchell T 2000 *Proc. Natl. Acad. Sci. USA* 97: 5322-5327.
- Bolar J P, Norelli J L, Wong K W, Hays C K, Harman G E & Aldwinkle H S 2000 *Phytopathol.* 90: 72-77.
- Bolar J P, Norelli J L, Harman G E, Brown S K & Aldwinkle H S 2001 *Transgenic Res.* 10: 533-543.
- Brogliè K E, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais C J & Brogliè R 1991 *Science* 254: 1194-1197.
- Brunner K, Peterbauer C K, Mach R L, Lorito M, Zeilinger S & Kubicek C P 2003 *Curr. Genet.* 43: 289-295.
- Brunner K, Zeilinger S, Ciliento R, Woo S L, Lorito M, Kubicek C P & Mach R L 2005 *Appl. Environ. Microbiol.* 71: 3959-3965.
- Cabib E 1987 *Adv. Enzymol.* 59: 59-101.
- Carsolio C, Gutierrez A, Gimenez B, Van Montagu M & Herrera-Estrella A 1994 *Proc. Natl. Acad. Sci. USA* 91: 10903-10907.
- Carstens M, Vivier M A & Pectorius I S 2003 *Transgenic Res.* 1150: 1-12.
- Chemin L S, Ismailov Z, Haran S & Chet I 1995 *Appl. Environ. Microbiol.* 61: 1720-1726.
- Chemin L & Chet I 2002 In: *Enzymes in the Environment - Activity, Ecology and Applications*. Marcel Dekker, New York, pp. 171-226.
- Chet I & Inbar J 1994 *Appl. Biochem. Biotechnol.* 48: 37-43.
- Chet I & Inbar J 1997 In: *Fungal Biotechnology*. Chapman and Hall, Weinheim.
- Cohen E 1993 *Arch. Insect Biochem. Physiol.* 22: 245-261.
- Cortes C, Gutierrez A, Olmedo V, Inbar J, Chet I & Herrera-Estrella A 1998 *Mol. Gen. Genet.* 160: 218-225.

- De Block M, Brouwer D D & Tenning P 1989 *Plant Physiol.* 91: 694-701.
- De la Cruz J & Llobell A 1999 *Eur. J. Biochem.* 265: 145-151.
- De la Cruz J, Hidalgo-Gallego A, Lora J M, Benitez T, Pintor-Toro J A & Llobell A 1992 *Eur. J. Biochem.* 206: 859-867.
- De la Fuente-Martinez J M & Herrera-Estrella L 1993 *Agric. Biotech News and Information* 5: 595-599.
- Deane E E, Whipps J M, Lynch J M & Peberdy J F 1998 *Biochemica and Biophysica Acta* 1353: 101-110.
- Draborg H, Kaupinnen S, Dalboge H & Christgau S 1995 *Biochem. Mol. Biol. Interact.* 36: 781-791.
- Elad Y & Kapat A 1999 *Eur. J. Plant Path.* 105: 177-189.
- El Quakfaoui S, Potvin C, Brazezinski R & Asselin A 1995 *Plant Cell Rep.* 15: 222-226.
- El-Katatny M H, Gudelj M, Robra K H, Elnaghy M A & Gubitz G M 2001 *Appl. Microbiol. Biotechnol.* 56: 137-143.
- El-Tarabily K A, Soliman M H, Nassar A H, Al-Hassani H A, Sivachidhambaram K 2000 *Plant Pathol.* 49: 573-583.
- Fakhouri W, Walker F, Armbruster W & Buchenauer H 2003 *Physiol. Mol. Plant Pathol.* 63: 263-269.
- Fang W, Leng B, Xiao Y, Jin K, Ma J, Fan Y, Feng J, Yang X, Zhang Y & Pei Y 2005 *Appl. Environ. Microbiol.* 71: 363-370.
- Fenice M, Selbmann N, Di Giambattista R & Federici F 1998 *Microbiol. Res.* 149: 289-300.
- Fogliano V, Ballio A, Gallo M, Woo S L, Scalla F & Lorito M 2002 *Mol. Plant-Microbe Interact* 15: 323-333.
- Folders J, Algra J, Roelofs, M S, Van Loon, L C, Tommassen J & Bitter W 2001 *J. Bacteriol.* 183: 7044-7052.
- Frankowski J, Lorito M, Scala F, Schmid R, Berg G & Bahl H 2001 *Arch. Microbiol.* 176: 421-426.
- Fuchs M, Tricoli D M, Carney K J, Schesser M, McFerson J R & Gonsalves D 1998 *Pl. Dis.* 82: 1350-1356.
- Gadoury D M, Machardy W E & Rosenberger D M 1989 *Pl. Dis.* 73: 98-105.
- Garcia I, Lora J M, De la Cruz J, Benitez T, Llobell A & Pintor-Toro JA 1994 *Curr. Genet.* 27: 83-89.

- Germia R, Goldman G H, Jacobs D, Ardles W, Vila S B, Van Montagu M & Herrera-Estrella A 1993 *Mol. Microbiol.* 8: 603-613.
- Giczey G, Kerenyi Z, Dallmann G & Hornok L 1998 *FEMS Microbiol. Lett.* 165: 247-252.
- Goldman G H, Hayes C & Harman G E 1994 *Trends Biotechnol.* 12: 478-482.
- Gooday G 1990 *Microbiol. Ecol.* 10: 387- 431.
- Govindsamy V, Gunaratna K R, Balasubramanian R 1998 *Can. J. Plant Pathol.* 20: 62-68
- Grinyer J, Hunt S, McKay M, Herbert B R & Nevalainen H 2005 *Curr. Genet.* 47: 381-383.
- Haran S, Schickler H & Chet I 1996 *Microbiol.* 142: 2321-2331.
- Haran S, Schikler H, Oppenheim A & Chet I 1995 *Mycol. Res.* 99: 441-446.
- Harman G E, Hayes C K, Lorito M, Broadway R M, Di Pietro A & Tronsmo A 1993 *Phytopathol.* 83: 313-318.
- Hayes C K, Klemsdal S, Lorito M, Di Pietro A, Peterbauer C, Nakasa J P, Tronsmo A & Harman G E 1994 *Gene* 138: 143-148.
- Howie W, Joe L, Newbiggin E, Suslow T & Dunsmuir P 1994 *Transgenic Res.* 3: 90-98.
- Inbar J & Chet I 1991 *Soil Biol. Biochem.* 23: 973-978.
- Inbar J & Chet I 1995 *Microbiol.* 141: 2823-2829.
- Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J & Mass C 1995 *Plant J.* 8: 97-109.
- Jach G, Logemann S, Wolf G, Oppernhem A, Chet I, Schell J & Logemann J 1992 *Biopractice* 1: 33-40.
- Jinzhua S, Qian Y, Beidong I & Dianfu C 2005 *Appl. Microbiol. Biotechnol.* Apr 5 [Epub ahead of print].
- Jolles P & Jolles J 1984 *Biochem.* 63: 165-189.
- Jones JD G, Dean C, Gidoni D, Gilbert D, Bond-Nutter D, Lee R, Bedbrook J & Dunsmuir P 1988 *Mol. Genet.* 212: 536-542.
- Kapat A, Zimand G & Elad Y 1998 *Physiol. Mol. Plant Pathol.* 52: 127-137.
- Keller H, Pamvoukdjian N, Ponchet M, Poupet A, Delon R, Verrier J L, Roby D & Ricci P 1999 *Plant Cell* 11: 223-235.
- Kikkert J R, Ali G S, Wallace P G, Reisch B & Reustle G M 2000 *Acta Hort.*(Wagening.) 528: 297-303.

- Kim D J, Baek J M, Uribe P, Kenerley C M & Cook D R 2002 *Curr. Genet.* 40: 374-384.
- Kneusel R E, Schiltz E & Maternm U 1994 *A. J. Biol. Chem.* 269: 3449-3456.
- Knoester M, Van Loon L C, Van den Heuvel J, Henning J, Bol J F & Linthorst H J M 1998 *Proc. Natl. Acad. Sci. U.S.A.* 95: 1933-1937.
- Kubicek C P, Mach R L, Peterbauer C K & Lorito M 2001 *J. Plant Pathol.* 83: 11-23.
- Kullnig C M, Krupica T, Woo S L, Mach R L, Rey M, Benitez T, Lorito M, Kubicek C P 2001 *Mycol. Res.* 105: 769-772.
- Lamb R C, Aldwinckle H S, Way R D & Terry D E 1979 *Hort. Sci.* 14: 757-758.
- Limon M C, Pintor-Toro J A & Benitez T 1999 *Phytopathology* 89: 254-261.
- Lorito M & Scala F 1999 *J. Plant Pathol.* 81: 73-88.
- Lorito M, Farkas V, Rebuffat S, Bodo B & Kubicek C P 1996 *J. Bacteriol.* 178: 6382-6385.
- Lorito M, Hayes C K, Di Pietro A, Woo S L & Harman G E 1994a *Phytopathology* 84: 398-405.
- Lorito M, Harman G E, Hayes C K, Broadway R M, Tronsmo A, Woo S L & Di Pietro A 1993 *Phytopathology* 83: 302-307.
- Lorito M, Peterbauer C K, Hayes C K, Woo S L & Harman G E 1994b *Microbiology* 140: 623-629.
- Lorito M, Woo S L, Garcia I, Colucci G, Harman G E, Pintor-Toro J A, Filippone E, Muccifora S, Lawrence C B, Zoina A, Tuzun S & Scala F 1998 *Proc Natl. Acad. Sci. (USA)* 95: 7860-7865.
- Malathi P, Viswanathan R, Padmanaban P, Mohanraj D & Ramesh Sundar A 2002 *Curr. Sci.* 83: 745-749.
- Marco L.D., Valadares-Inglis M.C. & Felix C.R. 2004 *Appl. Microbiol. Biotechnol.* 64: 70-75.
- Mathivanan N, Kabilan V, Murugesan K 1998 *Can. J. Microbiol.* 44: 646-651.
- Melentev A I, Aktuganov G E, & Galimxyanova N F 2001 *Microbiology* (Engl. Trans. Mikrobiologia) 70: 548-552.
- Merwin I A, Brown S K, Rosenberger D A, Cooley D R & Berkett L P

- 1994 *Pl. Dis.* 78: 4-10.
- Mourgues F, Brisset M N & Chevrau E 1998 *Trends in Biotechnol.* 16: 203-210.
- Murray F, Llewellyn D, McFadden H, Last D, Dennis E.S & Peacock W L 1999 *Mol. Breed.* 5: 219-232.
- Neilsen M N, Sorenson J, Fels J & Pedersen H C 1998 *Appl. Environ. Microbiol.* 64: 3563-3569.
- Ordentlich A, Elad Y, & Chet I 1998 *Phytopathol.* 78: 84-88.
- Panopoulous M T & Peet R C 1985 *Annu. Review Phytopathol.* 23: 381-419
- Peterbauer C K, Loroto M, Hayes C K, Harman G E & Kubicek C P 1996 *Curr. Genet.* 30: 325-331.
- Reguera G & Leschine S B 2001 *FEMS Microbiol. Lett.* 204: 367-374.
- Ren Y & West C A 1992 *Plant Physiol.* 99: 1169-1178.
- Roberts W A & Selitrennikoff C P 1998 *J. Gen. Microbiol.* 134: 169-176.
- Schmidt C S, Lorenz D & Wolf G A 2001 *J. Phytopathol.* 149: 427-435.
- Shanmugam V, Sriram S, Babu S, Nandakumar R, Raghuchandar T, Balasubramaniam P & Samiyappan R 2001 *J. Appl. Microbiol.* 90: 320-329.
- Shapira R, Ordentlich A, Chet I & Oppenheim A B 1989 *Phytopathol.* 79: 1246-1249.
- Sindhu S S & Dadarwal K R 2001 *Microbiol. Res.* 156: 353-358.
- Sriram S, Raghuchandar T, Babu S, Nandakumar R, Shanmugam V, Balasubramaniam P, Muthukrishnan S & Samiyappan R 2000 *Can. J. Microbiol.* 46: 520-524.
- Suslow T V, Matsubara D, Jones J, Lee R & Dunsmuir P 1988 *Phytopathol.* 78: 1556.
- Tepfer D, Boutteaux C, Vigon C, Aymes S, Perez V, O'Donohue M J, Huet J C & Pernollet J C 1998 *Mol. Plant-Microbe Interact.* 11: 64-67
- Terakawa T, Takaya N, Horiuchi H, Koike M & Takagi M 1997 *Pl. Cell Rep.* 16: 439-443.
- Thangavelu R, Palaniswamy A, Ramakrishnan G, Sabitha Duraiswamy, Muthukrishnan S & Velazhaghan R 2001 *J. Plant Dis. Protect.* 108: 433-445.

- Toyoda H, Katsuragi K, Tamai T & Ouchi S 1991 *Phytopathol. Zeit.* 133: 265-277.
- Ulhoa C J & Peberdy J F 1992 *Enzyme Microb. Technol.* 14: 236-240.
- Utisumi R, Hadama T, Noda M, Toyoda H, Hashimoto H & Ouchi S 1988 *J. Biotechnol.* 8: 311-316.
- Utisumi R, Yagi T, Katayama S, Katsuragi K, Tachibana K, Toyoda H, Ouchi D, Obata K, Shibano Y & Noda M 1991 *Agric. Biol. Chem.* 55: 1913-1918.
- Vaidya R J, Shah I M, Vyas, P R & Chatpar H S 2001 *World J. Microbiol. Biotechnol.* 17: 691-696.
- Vasseur V, Arigoni F, Andersen H, Defago G, Bompeix G & Seng J M 2001 *J. Gen. Microbiol.* 136: 2561-2567.
- Verberne M C, Verpoorte R, Bol J F, Mercado-Blanco J & Linthorst H J M 2000 *Nat. Biotechnol.* 18: 779-783.
- Vidyasekaran P 1997 Fungal pathogenesis in plants and crops: Molecular biology and host defense mechanisms. Marcel Dekker, New York. Pp.553
- Vidyasekaran P, Paranidharan V & Ramanathan A 1997 In: *Proc. Int. Program on rice Biotechnology*, Malacca, Malaysia. P.212
- Viswanathan R 2006 In: *Current status of Biological Control of Plant Diseases Using Antagonistic Organisms in India*, PDBC, Bangalore, pp. 304-309.
- Viswanathan R & Samiyappan R 2000 *Sugar Tech.* 2: 26-29.
- Viswanathan R & Samiyappan R 2001 *Microbiological Res.* 155: 309-314.
- Viswanathan R & Samiyappan R 2002 *Crop Protection* 21: 1-10.
- Viswanathan R, Ramesh Sundar A & Premkumari S M 2003a *World J. Microbiol. Biotechnol.* 19: 953-959.
- Viswanathan R, Premkumari S M & Ramesh Sundar A 2003b In: *6th International PGPR Workshop*, Oct 5-10, Calicut.
- Viswanathan R, Premkumari S M & Ramesh Sundar A 2004 In: *Proc. National Symposium on Frontiers in Biotechnology*, Oct. 27-28, Coimbatore (pp.68) Bharatiyar University, Coimbatore.
- Viswanathan R, Premkumari S M, Ramesh Sundar A & Kathiresan T 2006 *Curr. Sci.* 91: 951-956.

- Viterbo A, Haran S, Friesem D, Ramot O & Chet I 2001 *FEMS Microbiol. Lett.* 200: 169-174.
- Vyas P R & Deshpande M V 1989 *J. Appl. Gen. Microbiol.* 35: 343-350.
- Walker M J, Birch R G & Pemberton J M 1988 *Mol. Microbiol.* 2: 443-454.
- Wenzel G 1985 *Ann. Rev. Phytopathol.* 23: 149-172.
- Wong K W, Harman G E, Norelli J L, Ggustafson H L & Aldwinckle H S 1999 *Acta Hort.* 484: 595-599.
- Woo S L, Scala F, Ruocco M & Lorito M 2006 *Phytopathol.* 96: 181-185.
- Wu G, Shortt B J, Lawrence E B, Levine E B, Fitzsimmons K C & Shah D M 1995. *Plant Cell* 7: 1357-1368.
- Wu G, Shortt B J, Lawrence E B, Leon J, Fitzsimmons K C, Levine E B, Raskin I & Shah D M 1997 *Plant Physiol.* 115: 427-435.
- Zeilinger S, Galhaup C, Payer K, Woo S L, Mach R L, Fekete C, Loreto M & Kubicek C P 1999 *Fungal Genet. Biol.* 26: 131-140.
- Zhang L & Birch R G 1997 In: *Proc. Natl. Acad. Sci. USA.* 94: 9984-9989.
- Zimand G, Elad Y & Chet I 1996 *Phytopathol.* 86: 1255-1260.

Microbial control of insect pests

S Devasahayam

Microorganisms are associated with insects in several ways. They are useful as food of insects, in decomposing complex food materials into simpler substances for easy digestion and assimilation, in providing nutrients which are deficient in insect food, and also in causing diseases. The use of microorganisms pathogenic to insects or by their products in insect pest control is termed as microbial control. Microbial control forms an important component in integrated management of insect pests of many crops across the world.

History

Though the term microbial control was first suggested by Steinhaus in 1949, the idea of utilizing microbes for the control of insect pests however, had a very early beginning. The fact that insects (especially honey bee and silkworm) are affected by diseases was known even during the time of Aristotle. Bassi in 1935 demonstrated the infectious nature of the white muscardine fungus *Beauveria bassiana* on silkworm and other insects. The concept of utilizing a disease to control insect pests was first shown by Metchnikoff in 1879 who demonstrated that the grubs of the beetle *Anisoplia austriaca* can be infected with the green muscardine fungus *Metarhizium anisopliae*. The first commercial product of a microbial pesticide, Sporeine, containing *Bacillus thuringiensis* was produced during the late 1930s in France.

Groups of insect pathogens

Viruses

Viruses have been isolated and described from more than 800 species of insects. Insect viruses are broadly classified as inclusion viruses and non-inclusion viruses. Inclusion viruses may be polyhedral or granular and the polyhedral virus may be called nuclear or cytoplasmic according to its site of multiplication. Nuclear polyhedrosis viruses are more common in Lepidoptera, Hymenoptera and Diptera and granular inclusion viruses in Lepidoptera. Insect viruses are particularly suited to low input local production units and though labour intensive, do not require high-cost capital investment.

Bacteria

The bacterial pathogens, which affect insects, are broadly classified as spore formers and non-spore formers. There are both obligate spore formers like *Bacillus popilliae* and facultative spore formers like *B. thuringiensis*. About 100 species of entomopathogenic bacteria have been described among which *B. thuringiensis* is well known. Various commercial formulations of the bacteria are now available that are widely used for the management of various insect pests in the world. The most widely used biopesticides are those based on *B. thuringiensis*. Wider use of *B. thuringiensis* in developing countries not only depends on effective control and environmental safety aspects but also on reduced cost effective procedures for their manufacture.

Fungi

Fungi form the largest group of insect pathogens and more than 500 species have been reported from different insect hosts belonging to Lepidoptera, Coleoptera, Homoptera, Hymenoptera and Diptera. In most of the cases the adults are more commonly affected than the larvae. Various fungal pathogens are being commercially produced, the most common being *Beauveria bassiana* and *Metarhizium anisopliae*. Entomopathogenic fungi have been exploited on a reasonably large scale in many developing countries in predominantly local production units utilizing agricultural byproducts.

Protozoa

Protozoan pathogens belonging to the Class Sporozoa contain several pathogens infecting Lepidoptera, Coleoptera, Orthoptera, Hemiptera and Diptera. However, the role of protozoa as microbial agents in insect pest management is limited due to their difficulty in mass multiplication and field application and no commercial use has been reported.

Characteristics of insect diseases

Pathogenicity

Pathogenicity is the ability of a strain of microorganism to produce diseases in various hosts. Virulence is the pathogenicity of microorganism against a specific host under controlled conditions within a group or species of microorganisms. Infectivity is the capacity of the pathogen to spread from one host to another.

Transmission

Insect diseases are transmitted in various ways among which ingestion of an infective stage of the insect pathogen is the most common

Some insect pathogens such as fungi can directly enter the insect body through the cuticle and also through body openings of the insect. Many insect pathogens can be transmitted from an infected female via the egg to her offspring. Certain insect pathogens may also be transmitted from one insect host to another through hymenopterous parasites, which carry the pathogens on their ovipositor and introduce them into other insect hosts during oviposition.

Host range

Some insect pathogens are host specific and infect only one or very few insect species the same family. Some of the insect viruses are restricted to insect species of the same genus, while other insect viruses infect insect species of other families and occasionally different orders. Other insect pathogens have a very broad host range capable of infecting many insect species. The bacterium *B. thuringiensis* infests larvae of more than 500 species of insects belonging to Lepidoptera, Diptera, Hymenoptera, Coleoptera and other orders. Generally entomopathogenic fungi have a wide host range infecting many insect species belonging to different orders.

Persistence

Most insect pathogens have evolved resistant stages in their life cycle that persist in soil, foliage, faeces or cadaver of insects. Spore-forming bacteria are capable of persisting in the environment for many years outside the host. Polyhedra of the nuclear polyhedrosis virus of some insects can survive in soil for over 5 years. However, certain non-inclusion insect viruses can persist outside the host for a few weeks only. The persistence of the pathogen especially outside its host and its pathogenicity is dependent on environmental factors such as sunlight, relative humidity and temperature. Higher temperatures decrease the infectivity of insect pathogens especially viruses. Higher humidity is ideal for the multiplication and development of fungal pathogens.

Symptoms of insect diseases

Most insect diseases cause typical behavioral changes and disorders in insects peculiar to the disease. The affected insects may be markedly restless and irritable during early stages of the disease. Later they become lethargic and sluggish in their movement after the disease is well developed and may become moribund devoid of all movements. The affected insects also assume characteristic coloration distinguishing them from healthy insects. The change in coloration may be uniform over the entire body or only in spots or blotches due to changes within the affected tissues or due

to the presence of the organisms themselves. The infected insects also undergo physiological disturbances such as regurgitation of food, discharge of fluid from the anus and lack of sensitivity to heat, light and touch. Changes in consistency of body tissue and fluid are also seen in some infections and the tissue may thicken or liquefy and the body may become cloudy or milky. The body wall may rupture and fluid may ooze out giving a putrefying odour. The chitinous cuticle may also become brittle. The various symptoms depend upon the type of insect pathogen affecting the insect.

Diagnosis of insect diseases

Collection of dead specimens

The dead insect specimens should be collected in vials with a part of the substrate to avoid loss of essential symptoms of infection. Accumulation of excessive moisture inside the vial should be avoided. All materials should be transported without exposure to extremes of temperatures and stored in the refrigerator until further study.

Examination of pathogenic symptoms

The dead insect specimens are to be examined for gross symptoms and the group of insect pathogens causing the disease may be tentatively identified.

Viruses

The dead larvae may be found hanging or lying on a leaf with no filamentous structures on the cuticle. The cuticle may be fragile rupturing easily when disturbed and releasing the body contents, which become liquefied. Sometimes, white masses of fat body may be visible through the cuticle and the host body may take a bluish iridescence.

Bacteria

The body of the dead insect may be soft or dried to a scale and dark in colour with dark body fluid and tissues emitting a putrid odour. However, in certain cases the body may be white or reddish depending on the pathogen infecting the insect. Some lepidopteran caterpillars may be found hanging by their prolegs from plants.

Fungi

Body mummified, hard and does not integrate in water and sometimes filled with or covered with filamentous hyphae with tufts of hyphae growing out between segments. The affected insects may be found clinging to stems of plants with legs wrapped around or stuck to substrate with a white halo of spores on the surface surrounding them.

Protozoa

Body soft and breakable or dried to a scale that yields a milky mass in water.

Examination of tissues

Since there is much of overlapping and similarity in gross symptoms caused by various groups of pathogens, diagnosis of a particular group requires microscopic examination of tissues and body fluids as wet mount or smear or as histological sections. The dissecting microscope and the compound research microscope are essential tools in diagnosing diseases of insects. A phase contrast microscope would enable viewing of structures such as bacterial spores, inclusion viruses and protozoans more clearly without staining. However, an electron microscope may be required for diagnoses of viruses.

Isolation, culture and pathogenicity tests

The common method of isolating inclusion viruses is to keep the infected larvae in a vial with distilled water for a few days and the inclusion bodies will accumulate as a white layer on the bottom of the tube. The inclusion bodies can be separated from other contaminants by filtration and centrifugation and stored under refrigeration. For isolation of bacteria, the diseased insects are sterilized externally with alcohol, and rinsed in sterile distilled water. The specimens are dissected and the blood and body fluids are streaked in nutrient agar medium. The agar plates are incubated at room temperature overnight and the bacteria isolated in pure culture and maintained. For isolation of fungal pathogens, the diseased insect is surface sterilized in alcohol, rinsed in sterile water and the specimen broken and the pieces placed on PDA or suitable media. The plates are incubated at 20–25°C and observed for growth and sporulation and pure cultures are maintained. Pathogenicity tests are then performed, preferably on the same species of insect and Koch's postulates are to be proved to obtain conclusive evidence on the insect pathogen.

Advantages of microbial control

Microbial control of insect pests does not result in toxic residues and is highly specific so that beneficial insects and other non-target organisms are not affected when compared to chemical pesticides provided basic precautions have been undertaken. Insect pathogens are compatible with chemical pesticides and can be easily incorporated in integrated pest management schedules since they are compatible with other means of control such as host plant resistance, cultural control and biological control. The development of resistance by the host to insect pathogens is also slow in most cases. A desirable feature of microbial pathogens is that they are capable of reproduction and have the potential to multiply themselves in

the field. A large number of microbial pathogens are easy to mass-produce on natural substrates, which are cheaply available and also provide avenues of employment for rural people. Moreover the production processes depend on lower capital though labour intensive making them ideal for developing countries.

Disadvantages of microbial control

The high degree of specificity to the host makes them ecologically and environmentally attractive, but their narrow host range prevents their successful use in situations where when a complex of pests are to be controlled on a crop. The timing of application of microbial pathogens is also critical and sometimes difficult to judge. The efficacy of many microbial pathogens varies with climatic conditions and is influenced by sunlight, temperature, humidity, rainfall substrate and habitat. Though culturing of most insect pathogens is easy, they are difficult to store in virulent and viable conditions for a long period.

The present scenario

The market for microbial pesticides is growing though it represents less than 1% of the total crop protection market most of which is accounted for by Bt based products. The estimated market for biopesticides is around US\$ 500 million worldwide. At present biopesticides have a competitive advantage in small markets where competition from conventional chemicals is limited. In India use of microbial pathogens is on the increase and many small entrepreneurs have taken up its manufacture. However, many of them have little or no quality consciousness leading to lack of faith among the farmers in adopting microbial pathogens for the management of insect pests.

There are tremendous opportunities for genetic improvement of microbial pathogens for increased virulence, persistence, host range, stability in storage and amenability for low cost mass production, which would lead to their increased adoption in pest management schedules.

Keyword Index

A

- AFLP 18, 19, 135, 147, 259
- Allozymes 124, 125, 126, 127
- Antagonistic microbes 225, 227
- Antagonists 213, 216, 227, 228, 232, 233, 238
- Antibiosis 213, 215, 207, 225
- Antibiotics 48, 82, 215, 225, 234, 239, 242
- Antibody
 - antibody 4, 5, 6, 7, 72, 73, 74, 75, 76, 77, 166, 167, 168, 169, 170, 171, 173, 174, 175, 176, 177, 178, 201
- Antigen4, 6, 7, 72, 73, 74, 75, 76, 165, 166, 167, 168, 169, 170, 171, 173, 174, 175, 176, 177, 178, 201
- ARDRA 90, 91, 93, 144
- Autapomorphies 150, 259

B

- Bacteria
 - 17, 21, 45, 46, 47, 48, 49, 51, 53, 55, 57, 59, 61, 80, 101, 118, 120, 121, 122, 127, 141, 142, 143, 157, 160, 163, 170, 189, 217, 219, 227, 239, 253, 254, 256
- bacterial resistance 26, 259
- Beauveria bassiana*
 - 217, 219, 222, 252, 253
- Bioassays 174, 224
- Biocontrol 17, 195, 205, 207, 225, 226, 227, 228, 229, 231, 232, 236, 241, 242, 243
- biocontrol agents
 - 65, 134, 135, 186, 187, 207, 209, 211, 213, 215, 217, 219, 221, 223, 238, 241

Bioinformatics

- 33, 34, 35, 37, 110, 112, 156, 157, 159, 186, 187, 189, 190, 194
- Biological databases 34
- Black pepper 137, 139, 184
- BLAST 19, 20, 35, 36, 37, 38, 40, 110, 113, 234
- Burrowing nematode 118, 183, 184

C

- Cell wall degrading enzymes
 - 227, 241, 242
- Chitinase
 - 11, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 242
- Competent cells 82, 234
- CTAB method 64

D

- DAC-ELISA 72, 73, 74, 201
- Databases
 - 34, 35, 36, 97, 98, 99, 157, 163, 186, 187, 189, 191
- Diagnosis
 - 3, 4, 7, 8, 9, 97, 117, 118, 122, 123, 153, 155, 156, 157, 165, 166, 167, 168, 170, 179, 180, 203, 225, 256
- Diagnostics
 - 3, 123, 133, 155, 156, 157, 160, 161, 164, 170, 179, 186, 191, 194
- Dilution plate method 208
- Dimers 106, 107
- Direct ELISA 4, 72
- Directional cloning 78
- DNA viruses 180
- Dot immunobinding assay 75

DOT-ELISA 5
Dual culture method 213

E

ELISA
4, 5, 6, 7, 8, 10, 72, 73, 74, 76,
77, 158, 170, 173, 174, 176, 177, 180
Entomopathogenic fungi
216, 217, 218, 219, 224, 253,
254
EST clustering 39
Exotic pests 196, 197

F

FASTA 35
FISH 159
fish 101, 150
Fungal pathogens
9, 16, 25, 26, 120, 121, 134,
165, 216, 222, 225, 226, 234,
240, 253, 254, 256

Fungi

13, 45, 46, 47, 48, 49, 50, 55,
57, 64, 66, 89, 117, 118, 119,
120, 121, 135, 149, 155, 157,
158, 164, 165, 170, 179, 189,
200, 207, 209, 213, 214, 215,
216, 217, 218, 219, 221, 224,
225, 226, 227, 229, 230, 231,
234, 235, 237, 241, 243, 253, 254,
255

G

Gel electrophoresis 9, 63, 87,
91, 93, 126, 127, 128,
132, 133, 139, 142, 161
Gene chip 95
Gene cloning 138, 225
Gene mapping 18
Gene prediction 39
Genetic diversity 21, 134, 137
Genomics 13, 15, 160, 161, 162,
163, 164, 243

H

Hairpins 108

I

Image analysis 99
Indirect ELISA 4, 72, 75
Information Technology 157,
193, 194, 195
Insect pathogens 217, 222, 252,
253, 254, 255, 256, 257
Internally transcribed spacer
see ITS
Isolation of DNA 64, 65, 66
Isozymes 103, 104, 124, 125, 126,
127, 130, 133, 135
ITS
98, 134, 137, 138, 147, 183, 185

L

Ligation 78, 80, 82, 137, 138

M

Mass spectrometry
161, 163, 164, 189
Maximum likelihood 112, 113,
150, 151
Microarrays
95, 96, 97, 100, 101, 160, 161, 162
Molecular cloning 11, 83, 137, 140
Molecular diagnostics 4, 64
Molecular indexing 8
Molecular markers 4, 18, 21, 88,
89, 127, 147
Monoclonal antibodies 3, 7, 74,
122, 158, 166, 167,
171, 174, 201
Monophyletic taxon 149
Multiple sequence alignment
37, 40, 109, 110, 112, 113

N

Neighbor-joining 112, 150

O

Operational taxonomic units 148
Orthologs 148

P

PAGE 87, 107, 128, 129, 141,
229, 230, 233, 234, 238
Paraphyletic taxon 150
Parsimony 112, 150, 151
Pathogenicity
10, 11, 120, 237, 238,
241, 253, 254, 256
PCA 38, 50, 100, 219
PCR 3, 9, 10, 78, 79, 80, 83, 84,
85, 87, 90, 91, 92, 93, 96, 97,
98, 100, 106, 108, 109, 110, 134,
135, 136, 138, 139, 140, 142, 143,
144, 158, 159, 160, 162, 163,
164, 180, 181, 182, 184, 186
PCR amplification
90, 92, 108, 135, 138, 143,
158, 160, 184
Peroxidase 103, 104, 132, 174, 177
Pest risk analysis 156, 197
Phylogenetic analysis
90, 141, 148, 150
Phylogenetics 146, 147, 151
Phylogeny
40, 113, 141, 146, 148, 150, 187
Phytopathogen
10, 225, 226, 240, 241, 242
Phytophthora
13, 16, 19, 20, 25, 49, 51, 60,
65, 137, 138, 155, 157, 170,
194, 202, 242
Plant disease resistance 10, 19
Plant pathogens 17, 65, 72, 77,
95, 117, 123, 157, 158,
159, 161, 162, 163, 164, 165, 171,
172, 178, 179, 187, 195, 196,
199, 213, 216, 225, 226,
230, 237

Plant quarantine 156, 159, 196,
197, 198, 199, 202
Plasmid vector 80
Pleisiomorphies 150
Polyphyletic taxon 150
Primer 38, 78, 84, 85, 86, 87,
88, 89, 91, 92, 93, 98, 106, 107,
108, 109, 110, 111, 134, 135, 136,
138, 180, 181, 185, 186, 189
Primer designing 106
Proteomics
160, 161, 163, 164, 189,
243, 244
Protozoa 45, 216, 224, 253, 256

Q

QTLs 19, 20

R

R genes 10, 11, 12, 14, 16, 18
Radopholus similis 183
RAPD
18, 19, 84, 85, 86, 87, 88,
89, 136, 142, 144, 147
rDNA
90, 91, 93, 97, 98, 101, 134,
137, 144, 183, 185
Real time PCR 158, 162
Rep-PCR 143, 158
RFLP
19, 88, 135, 136, 141, 142, 144, 159
RFLP PCR 138
Ribosomal DNA 90, 44, 183, 187
RNA viruses 181
RT-PCR
3, 9, 10, 163, 164, 181, 182, 234

S

SDS method 64, 66
Sequence analysis 33, 35,
137, 144, 187
Serology 157, 165, 166, 180
Single nucleotide polymorphism
127, 188

SOD 104, 132
Synapomorphies 150
Systematics 146, 147, 151

T

TA cloning 78
Taxonomy
 95, 98, 127, 137, 141, 146, 147,
 151, 187, 191, 192,
 194, 195, 211, 224
Transcriptome 95
Transformation 17, 21, 25,
 80, 81, 82, 83, 137, 138,
 225, 226, 234, 238
Transgenic plants
 16, 17, 18, 22, 23, 24, 26,
 27, 28, 234, 235
Transmission
 22, 117, 120, 122, 180, 201,
 253

Transmission electron microscope
 201

Trichoderma

57, 65, 66, 207, 208, 209, 210, 211,
224, 227, 228, 229, 231, 232,
240, 241, 242, 243, 244, 245

V

Verticillium lecanii 217, 222
Viral resistance 22, 24
Virus detection
 7, 163, 177, 178, 179
Viruses 4, 5, 6, 7, 8, 22, 68

X

Xenologs 148

