MOLECULAR CHARACTERIZATION OF TOMATO (*Solanum lycopersicum* L.) WITH SPECIAL REFERENCE TO TOMATO LEAF CURL VIRUS (ToLCV) RESISTANCE

By

ANJALI DIVAKARAN
(2005 - 11 - 140)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture
Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2007

DECLARATION

I hereby declare that the thesis entitled "**Molecular characterization of tomato (*Solanum lycopersicum* L.) with special reference to tomato leaf curl virus (ToLCV) resistance**" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Vellanikkara,
4-04-2008

Anjali Divakaran
(2005-11-140)

CERTIFICATE

Certified that the thesis entitled "**Molecular characterization of tomato (*Solanum lycopersicum* L.) with special reference to tomato leaf curl virus (ToLCV) resistance**" is a record of research work done independently by Ms. Anjali Divakaran (2005-11-140) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

Vellanikkara
4 -04-2008

Dr. P. A. Nazeem
(Major Advisor, Advisory Committee)
Professor and Head
Centre for Plant Biotechnology and
Molecular Biology (CPBMB)
College of Horticulture

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Anjali Divakaran (2005-11-140)**, a candidate for the degree of **Master of Science in Agriculture**, with major field in Plant Biotechnology, agree that the thesis entitled **"Molecular characterization of tomato (*Solanum lycopersicum* L.) with special reference to tomato leaf curl virus (ToLCV) resistance"** may be submitted by Ms. Anjali Divakaran in partial fulfillment of the requirement for the degree.

Dr. P. A. Nazeem
(Major advisor)
Professor and Head
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture
Vellanikkara

Dr. Sally K. Mathew
Professor
Department of Plant Pathology
College of Horticulture
Vellanikkara
(Member)

Dr. D. Girija
Professor
Centre for Plant Biotechnology and
Molecular Biology
College of Horticulture
(Member)

Dr. S. Nirmala Devi
Professor
Department of Olericulture
College of Horticulture
Vellanikkara
(Member)

24/3/08
External Examiner
Dr. M. Balasundram
Scientist-In Charge (Head) Biotechnology
Kerala Forest Research Institute
Peechi, Thrissur.

Acknowledgement

I wish to place on record the names of all the people who have helped me in the successful completion of my thesis work.

Let me start with **Dr. P. A. Nazeem,** *Professor and Head, CPBMB and chairperson of my Advisory Committee. Her expert guidance, constant encouragement, valuable suggestions and ideas and constructive criticism have played the major role in helping me all along the way. I am highly indebted to her for her ever-willing cooperation and great patience. I take this opportunity to express my respect and wholehearted gratitude to Nazeem Madam.*

I am truly grateful to **Dr. Sally K. Mathew,** *Professor, Department of Plant Pathology and member of my Advisory Committee for her well-timed suggestions, critical assessment throughout the period of the work and painstaking scrutiny of the manuscript. Her willingness to extend help*

Dr. D. Girija, *Professor, CPBMB and one among my Advisory Committee members helped me to a great extent by providing critical and timely help. I thank her for all the help and cooperation she has extended to me.*

I am extremely thankful to **Dr. S. Nirmala Devi,** *Professor, Department of Olericulture and member of my Advisory Committee, whose expertise and knowledge have been of immense help during field work.*

I am grateful to **Dr. Sadhan Kumar,** *Professor, Department of Olericulture for his advice and suggestions during the course of the project.*

I am extremely thankful to **Sri. S. Krishnan,** *Assistant Professor, Department of Agricultural Statistics, for the help rendered in statistical analysis of data.*

*I wish to express my sincere thanks to **Keshavachandran** sir, **Valsala** madam, **Augustin** sir, **Rajendran** sir and **Sujatha** madam for their cooperation and kindness throughout the course of the studies.*

*I express my hearfelt gratefulness to **Shiba** chechi, **Simi** chechi, **Shailaja** chechi, **Seena** chechi, **Firoz** chettan, **Praveena**, **Soumya**, **Sherin** and **Sheeba** of CPBMB, who helped me in several ways for the completion of this venture.*

*I thank **Mr. P. K. Sreekumar**, Farm Assistant, CPBMB, for the help in photography.*

*Special thanks go to **Santhosh** chettan, Students Computer Club, COH, for rendering necessary help whenever needed.*

*Special mention goes to **Shobha** chechi and **Omana** chechi without who the field work would not have gone so smoothly. I express my deep gratitude to them and the rest of the labourers of CPBMB.*

*I take this opportunity to thank my classmates and friends, **Geena**, **Sameera**, **Neema**, **Ramya**, **Likhitha**, **Liffey** and **Fayas** for their help and cooperation for the entire period of study.*

*I specially thank my friends **Saisree**, **Dana**, **Hima**, **Sangeetha**, **Mittu**, **Aravind**, **Nisha**, **Sameera**, **Hema**, **Swapna**, **Thenmozhi**, **Kimi**, **Renu**, **Sweta**, **Niharika**, **Sindhu**, **Jessna**, **Regina**, **Asha**, **Rashmi**, **Dinesh**, **Rahul**, **Thiagarajan** and **Satish** for their support and encouragement.*

*My heartfelt gratitude to my loving **parents** and **sister** for their constant prayers, affection, moral support and sincere encouragement at all times.*

*Above all, I humbly bow my head before the **Almighty**, who blessed me with willpower and courage to complete this endeavour successfully.*

Anjali Divakaran
(2005-11-140)

Dedicated to My Wonderful Parents and Sister

ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
cm	Centimeter
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene Diamine Tetra Acetic acid
FLA	Fluorescent Image Analyzing system
g	Gram
HARP	Horticulture and Agroforestry Research Project
HPKV	Himachal Pradesh Krishi Viswa Vidyalaya
IIVR	Indian Institute of Vegetable Research
KAU	Kerala Agricultural University
M	Mole
ml	Millilitre
mM	Millimole
µg	Microgram
µl	Microlitre
µM	Micromole
ng	Nanogram
nm	Nanometer
NTSyS	Numerical Taxonomy System of Multivariate Statistical Program
OD	Optical Density
OUAT	Orissa University of Agriculture and Technology
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
%	Percentage
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
rpm	Rotations per minute
SAHN	Sequential Agglomerative Hierarchical Nested Clustering
TAE	Tris Acetate EDTA
TE	Tris EDTA
TEMED	N, N, N, N- Tetramethylene ethylene diamine
ToLCV	Tomato Leaf Curl Virus
ToYLCV	Tomato Yellow Leaf Curl Virus
U	Unit
UPGMA	Unweighted Pair Group Method of Arithmetic Averages
V	Volts
v/v	Volume by volume
w/v	Weight by volume

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Introduction

1. INTRODUCTION

The solanaceae is one of the most economically important families among vegetable crops. Among the members of the solanaceae family, tomato (*Solanum lycopersicum* L.) is one of the major vegetable crops in the world. India with an area of 0.50 million hectares and with productivity of 17.4 MT/ha is the sixth largest producer of tomatoes in the world (Chamber *et al.*, 2006).

Nutritionally, tomato is a significant dietary source of Vitamin A and C. Furthermore, recent studies have shown the importance of lycopene, a major component of red tomatoes, which has antioxidant properties that may help to protect against human diseases such as cancer and heart disease.

Leaf curl caused by the Tomato Leaf Curl Virus (ToLCV), a heterogenous complex of whitefly-vectored geminivirus is a serious production constraint of tomato worldwide, particularly in the Indian subcontinent and many other Asian countries. The disease is spread through the whitefly *Bemisia tabaci.* The effect of the disease is near total loss of crops. Each year ToLCV causes millions of dollars damage to tomato crops all over the world. Sadashiva *et al.* (2006) have reported that the disease results in yield losses between 70 and 100 per cent.

Geminiviruses form the second largest family of plant viruses, the *Geminiviridae*, represented by four genera namely Mastrevirus, Curtovirus, Topocuvirus and Begomovirus. During the last two decades these viruses have emerged as devastating pathogens, particularly in the tropics and sub-tropics causing astronomic economic losses and threatening crop production. Compared to other virus groups of the *Geminiviridae* family, begomoviruses have emerged as more serious problems in a variety of crops especially tomato. Major contributory factors for the emergence and spread of these viruses are evolution of variants of the viruses and increase in vector population.

Adoption of disease resistant varieties/hybrids is the most practical way to combat the menace of ToLCV. Several ToLCV resistant tomato varieties and F_1 hybrids are being economically cultivated in India. However, none of them is resistant to all the prevalent ToLCV strains. Identification of resistant sources for the disease and isolation of resistance genes by the help of molecular markers linked to resistance followed by the pyramiding of these genes could be the most feasible way to overcome the problem of ToLCV.

The International Sol Genome project, including more that 30 countries all over the world was initiated with the long-term objective of developing a web-based Solanaceae bioinformatics framework that will link sequences, phenotypes and habitats to promote scientific discovery. The SOL community is presently sequencing the tomato, potato and tobacco genomes through national grant as well as international collaborative projects. Among these, tomato has been selected as the reference species for genome sequencing. The Centre for Plant Biotechnology and Molecular Biology is one among the different centres around the country involved in the development of trait-related molecular markers and identification of resistance genes to ToLCV and bacterial wilt disease by systematic screening of tomato genotypes collected from different parts of India and characterization of the selected genotypes using molecular markers as part of the Sol Genome project. The thesis entitled "Molecular characterization of tomato (*Solanum lycopersicum* L.) with special reference to tomato leaf curl virus (ToLCV) resistance" was undertaken as part of this worldwide project.

The main objectives of the study were:

1. Screening of tomato genotypes already reported to be resistant/susceptible to leaf curl in regions other than Kerala, for their reaction to ToLCV under conditions prevalent in the state.
2. Molecular characterization of the selected tomato genotypes with reference to ToLCV resistance using RAPD and AFLP marker systems.

Review of Literature

2. REVIEW OF LITERATURE

A brief review of literature collected with reference to the importance of the crop, the virus and disease, resistant sources and molecular characterization is dealt with in this chapter.

2.1 Crop and the Disease

2.1.1 About the Crop

The cultivated tomato originated in wild form from the Peru-Ecuador-Bolivia area of the Andes (South America). The first known record of tomato is in the year 1554 in South America. Since 1800, tomatoes are being used as food all over the world. Tomato was originally named *Solanum lycopersicum* by Linnaeus. In 1754, Miller separated tomatoes and designated the genus *Lycopersicon* and the species *esculentum* for the cultivated tomato and *Lycopersicon pimpinellifolium* to the wild forms of tomato. Based on molecular and morphological information, a new taxonomic classification of tomato and readoption of *Solanum lycopersicum* for the cultivated tomato had been suggested a while ago (Foolad, 2007). Tomato has now been renamed as *Solanum lycopersicum* (Gupta *et al.*, 2006; Riccardia *et al.*, 2007).

The species included in the genus *Lycopersicon* are, *L. esculentum*, *L. pimpinellifolium*, *L. cheesmanii*, *L. hirsutum*, *L. chemelewskii*, *L. parvifolium*, *L. chinense*, *L. peruvianum* and *L. chilense* (Thamburaj and Singh, 2001).

2.1.2 Tomato Leaf Curl Virus Disease

A. Pathogen and Symptom

Tomato is affected by 30 different viruses belonging to 16 different taxonomic groups. Among them, the Geminivirus group, which causes leaf curl disease, is more frequently found in sub-tropical and tropical environments. In

tomato, leaf curl virus disease is an exhaustive one causing astronomic losses to the crop.

Geminiviruses form the second largest family of plant viruses. This family is represented by four genera : Mastrevirus, Curtovirus, Topocuvirus and Begomovirus.

During the last two decades these viruses have emerged as devastating pathogens, particularly in the tropics and sub-tropics, causing huge economic losses and threatening crop production (Varma and Malathi, 2003).

Tomato leaf curl virus (ToLCV) is a whitefly-transmitted (*Bemisia tabaci*) virus belonging to the family *Geminiviridae* and genus Begomovirus. Hussain (1932) was the first to report the leaf curl disease in tomato. Thung (1932) reported that the Tobacco Leaf Curl Virus causes leaf curl disease in tomato. In India, occurrence of leaf curl disease was first observed in the Northern plains by Pal and Tandon (1937) and later reported by Pruthi and Samuel (1939).

This destructive disease of tomato has been reported in many regions of India, East Asia and Australia (Thamburaj and Singh, 2001). Species of the genus Begomovirus are transmitted by the whitefly, *Bemisia tabaci*, in a persistent, circulative manner and infect dicotyledonous plants (Lapidot and Friedmann, 2002).

The yield loss accounted in tomato due to ToLCV infection ranges from 50 to 70 per cent (Gururaj *et al.*, 2002). The loss may be sometimes as high as 90 per cent and this varies with season and stages of crop growth at which the infection occurs (Saikia and Muniyappa, 1989).

The vector of ToLCV is a polyphagous insect with more than 300 hosts comprising of a lot of cultivated plants and weeds (Reddy *et al.*, 1986). It was

observed that the influence of ToLCV in tomato ranged from 17 to 53 per cent during July to November and up to 100 per cent during February to May (Saikia and Muniyappa, 1989).

The typical symptoms of the disease are leaf curling, yellowing, upward leaf rolling and bunched and stunted growth with distorted leaves in initial stages, which become more adverse in advanced stages (Kumar *et al.*, 2002).

Malathi (2006) reported that there are more than a hundred begomoviruses infecting tomato all over the world. Majority of begomoviruses occurring in New World (American) have bipartite genome, which consists of two components, referred to as 'DNA A' and 'DNA B'. DNA A encodes for encapsidation and replication and DNA B for movement functions. Begomoviruses infecting tomato in the Old World are monopartite. All functions required for viral pathogenesis are encoded in DNA A component.

B. Strains of ToLCV

Whitefly-transmitted tomato geminiviruses from southeast and East Asia constitute a cluster of geminiviruses distinct from those of the Middle East, southeastern Europe and America (Zeidan *et al.*, 1998).

Engel *et al.* (1998) reported that tomato infecting geminiviruses from Panama named Tomato Leaf Curl Virus (ToLCV-Pan) resembled other whitefly-transmitted geminiviruses, and produced the same kind of symptoms in tomatoes.

The presence of two subgroups of viruses causing tomato leaf curl disease in India on sequence analysis has been reported. Isolates belonging to subgroup I had a bipartite genome and were conserved among themselves, showing 94 to 95 per cent nucleotide sequence homology, while isolates belonging to the second

subgroup had monopartite genome and showed 73 to 75 per cent homology with subgroup I (Sinha *et al.*, 2004).

Malathi (2006) reported the presence of different strains such as a bipartite Tomato Leaf Curl New Delhi virus, a monopartite Tomato Leaf Curl Gujarat virus with a DNA B component, a Tomato Leaf Curl Bangalore virus with an additional satellite DNA B or DNA β component and a monopartite Tomato Leaf Curl Karnataka virus without any DNA B or DNA β component infecting tomato in the Indian subcontinent.

2.1.3 Screening for Disease Infection and Resistant Sources

In a study aimed at finding sources of resistance to ToLCV, which included screening of 122 varieties, lines and wild accessions of *Lycopersicon* for two years in three seasons, high degree of resistance was found in *Lycopersicon hirsutum* f. *glabratum* ('B6013') and *L. hirsutum* f. *typicum* ('A 1904'). In addition, five accessions of *L. peruvianum* were also found to be highly resistant (Banerjee and Kalloo, 1987).

Kasrawi *et al.* (1988) reported high levels of resistance in accession 'LA 385' of *L. peruvianum* f. *humifusum* and other accessions of *L. peruvianum* to tomato yellow leaf curl virus (TYLCV) and indicated their potential use in breeding programs.

Six lines, i.e., H-2, H-11, H-17, H-23, H-24, and H-36, resistant to Tomato Leaf Curl Virus (ToLCV) have been developed with controlled introgression of *L. hirsutum* f. *glabratum* into *Lycopersicon esculentum.* Line H-24 was found to be most resistant by showing least disease incidence and coefficient of infection values. The disease incidence, 120 days after inoculation, of all these lines varied from 8.3 to 35 per cent (Kalloo and Banerjee, 1990).

Zamir *et al.* (1994) reported the effect of gene *TY-1* with partial dominance mapped on chromosome 6 on TYLCV tolerance. This gene was mapped from the species *Lycopersicon chilense,* which is resistant to tomato yellow leaf curl virus.

Preliminary genetic studies indicated that tolerance to begomoviruses was controlled by one to five genes, some dominant and others recessive. Subsequent studies indicated that complete resistance to begomoviruses was controlled by a single dominant gene (Vidavsky and Czosnek, 1998).

Bhattacharjee (1999) identified promising segregants with high resistance to ToLCV and appreciable yield levels in tomato genotypes ATB-28, ATB-24 and ATB-94.

Resistance in *Lycopersicon hirsutum* f. *glabratum* ('B6013') to ToLCV was mapped at AVRDC, Taiwan to an introgression located at the lower end of chromosome 11 in the tomato genome (Hanson *et al.*, 2000).

Kalloo and Banerjee (2000) studied the performance of the genotype 'H-24', a resistant derivative obtained *via* modified backcross-pedigree selection from the cross *Lycopersicon esculentum* cv. Sel-7 x *Lycopersicon hirsutum f. glabratum* 'B6013'with respect to yield and reaction to ToLCV disease under field and artificial inoculation conditions. They reported that H-24 can be grown in leaf curl infested area and can be used as a tolerant breeding line.

In a study conducted at the Tamil Nadu Agricultural University, Coimbatore, involving eight hybrid and five parental lines of tomato in order to develop F_1 hybrids with resistance to ToLCV, Veeraragavathatham *et al.* (2002) reported that the parents MLCR 2, CLN 2123A and H-24 were considered better for ToLCV resistance and the hybrids CLN 2123A x MLCR 2 and MLCR 2 x CLN 2123A with ToLCV resistance were adjudged as high yielders.

8

The use of V-notch grafting to identify true ToLCV resistant plants in a bulk population of plants belonging to the genotype H-24 has been reported by Kumar *et al.* (2002). The true resistant plants identified were selfed and progeny further tested to develop pure lines resistant to ToLCV.

Gururaj *et al.* (2002) from Karnataka reported that the parental genotypes H-36, H-86 and NDT-VR-60 and three hybrids H-36 x H-86, H-36 x NDT-VR-73 and H-36 x L-15 were found to be resistant to ToLCV. The studies indicated positively significant correlation between whitefly population and ToLCV incidence.

Muniyappa *et al.* (2002) reported the origin, breeding and morphology of three tomato leaf curl virus (ToLCV) resistant tomato lines, namely TLB111, TLB130 and TLB182.

Singh *et al.* (2003) categorized different tomato genotypes as highly resistant, resistant, moderately resistant, susceptible, moderately susceptible and highly susceptible based on their reaction to ToLCV according to the score chart suggested by Banerjee and Kalloo (1998).

The resistance of accessions of *L. cheesmanii*, *L. pimpinellifolium* and *L. peruvianum* to ToLCV among a total of 90 genotypes of *Lycopersicon* species tested for resistance to ToLCV by agroinoculation and vector (*Bemisia tabaci*) inoculation techniques under insect-proof glasshouse conditions has been identified by Tripathi and Varma (2003).

Daniel (2003) reported that somaclones of *Lycopersicon pimpinellifolium*, and two somaclones of variety Sakthi were free from tomato leaf curl virus disease in a study conducted for the screening of tomato somaclones for resistance to ToLCV.

Narasegowda *et al.* (2003) reported that accessions of the wild species *Lycopersicon hirsutum* LA 1777 and PI 390659 were the best sources of resistance to both tomato yellow leaf curl virus, Israel (TYLCV-Is) and tomato leaf curl virus from Bangalore isolate 4, India (ToLCV-[Ban4]) in a study conducted to screen 34 tomato genotypes belonging to wild and domesticated lines for resistance to the two viruses.

Tripathi and Varma (2003) reported the use of agroinoculation and vector (*Bemisia tabaci*) inoculation techniques under insect-proof glasshouse conditions for a virus-resistance screening program involving a total of 90 genotypes of *Lycopersicon* species. The rate of infection in the inoculated plants was determined by detection of the viral DNA in individual plants by nucleic acid spot hybridization (NASH). Among 38 commercial cultivars screened, 42.1 per cent were highly susceptible in vector inoculations and 81.6 per cent in agroinoculation.

The variability of leaf curl resistance in tomato was assessed to select high yielding cultivars. The genotypes suited for future breeding programs based on high fruit setting, fruit size and disease reaction that were identified were Silvestra and TC 248307 (Singh *et al.*, 2003).

Brar and Singh (2003) screened 98 newly introduced exotic and indigenous cultivars of hot pepper (*Capsicum annuum* L.) against leaf curl and chilli mosaic viruses under natural epiphytotic conditions and found the genotypes IC 6, Sel. 217621-1, Sel. 217621-1-1-104 to 106, Sel. 217621-1-1-1-108 to 109 and Sel. 217621-1-1-1-113, Sel BT 1-3, Sel. BT-1-13, SE1 BT1-1-5, P404, P522, P846, U22-87 and MS-1 to be tolerant to both diseases.

Tavella *et al.* (2005) reported use of mechanical inoculation and grafting under laboratory conditions for artificial screening of tomato hybrids engineered with Tomato Spotted Wilt virus sequence for virus resistance.

Maruthi *et al.* (2005) reported low disease incidence (6-45%) in cultivars TLB111, TLB130, TLB133 and TLB182 resistant to South Indian ToLCV screened against Bangladesh ToLCVs.

Sadashiva *et al.* (2006) conducted a systematic screening work including field and controlled condition screening for the identification of stable sources of resistance to prevalent ToLCV strains. Out of the 45 tomato lines including four wild accessions with reported resistance to ToLCV evaluated, 39 resistant lines, three tolerant lines and three susceptible lines were obtained. All the 39 resistant lines were screened again in screen-house using viruliferous whiteflies for further confirmation of resistance.

Delatte *et al.* (2006) reported the use of graft inoculation and whitefly mediated inoculation to compare the reaction of two wild genotypes of *L. pimpinellifolium*, WVA106 (susceptible) and INRA-Hirsute (resistant) to tomato yellow leaf curl virus isolate 'Reunion'.

Complete resistance to ToLCV in eight tomato lines *viz*; IIHR-2101 (*Lycopersicon hirsutum* LA-1777), IIHR-2195, IIHR-2205, IIHR-2406, IIHR-2413, IIHR-2611 and two *Lycopersicon peruvianum* accessions (IIHR-1943 and IIHR-1970) after field screening and artificial inoculation using viruliferous whiteflies has been reported by Sadashiva *et al.* (2006). They also reported that susceptible check Pusa Ruby exhibited cent percent ToLCV incidence.

Bian *et al.* (2007) identified a tomato breeding line FLA653 that confers a high level of resistance to tomato leaf curl virus (ToLCV). Genetic analysis indicated that the resistance was controlled by a single recessive allele named *tgr-1*.

2.2 Molecular Characterization

In tomato, the development of a saturated RFLP map has facilitated mapping of several disease resistant genes through the use of molecular markers. These genes have been located throughout the tomato genome in different chromosomes (Young *et al.*, 1988).

Williams *et al.* (1990) developed a method (RAPD) that uses random primers in a polymerase chain reaction to rapidly generate polymorphic markers that can be used to create genetic linkage maps. They reported that RAPD is a dominant marker inherited in mendelian fashion.

Three markers generated by random primers polymorphic in Near Isogenic Lines (NILs) of tomato have been identified. These markers were reportedly linked to the *Pto* gene, which is supposed to confer resistance to the bacterial disease caused by *Pseudomonas* (Martin *et al.*, 1991).

Martin *et al.* (1993) reported the success of the map-based cloning strategy in the cloning of a disease resistance gene in tomato. The *Pto* gene that confers resistance to bacterial speck disease in tomato was cloned through a map-based strategy.

It has been reported that RAPD markers differ according to variations in experimental conditions. Wolf *et al.* (1993) reported that RAPD markers vary according to changes in concentration of $MgCl_2$ and the type of thermal cycler, while Schiewater *et al.* (1993) reported that the variation occurs with change in quantity and quality of *Taq* polymerase enzyme.

Many complications of a phenotype-based assay can be mitigated through direct identification of genotype with a DNA-based diagnostic assay. For this reason, DNA-based genetic markers are being integrated into several plant

systems and are expected to play an important role in the future of plant breeding. The RAPD assay can be used for a number of applications, which include development of genetic maps, targeting molecular markers, pooling of genomic DNA from individuals that are known to be fixed at a particular locus and study of individual identity and taxonomic relationship in both eukaryotic and prokaryotic organisms. (Tingey and del Tufo, 1993).

Chunwongse *et al.* (1994) reported the tagging of a powdery mildew resistance gene, *Lv*, in tomato using RAPD and RFLP markers. Screening was carried out with 300 random primers that were used to amplify DNA of resistant and susceptible cultivars.

Vos *et al.* (1995) described a novel technique for DNA fingerprinting, namely AFLP (Amplified Fragment Length Polymorphism). They illustrated how this technique can be used in fingerprinting of genomic DNA of λ-phage, Acinetobacter, yeast, *Arabidopsis*, cucumber, tomato, human, etc. In tomato, the reaction was carried out using the combination of *Eco*RI and *Mse*I enzymes. They reported that AFLP procedure is insensitive to template DNA concentration. According to their findings, AFLP is an effective tool to reveal polymorphism.

Chague *et al.* (1996) identified RAPD markers linked to *Sw-5* gene, which confers resistance to spotted wilt virus disease in tomato. They have identified four RAPD markers for *Sw-5*. Markers R2 and S are tightly linked to this gene. For RAPD analysis DNA was extracted from each plant of the segregating population and the parent cultivars as described by Dellaporta *et al.* (1983).

Haanstra *et al.* (1999) reported the development of an integrated high-density RFLP-AFLP map of tomato based on two *Lycopersicon esculentum* x *Lycopersicon pennellii* F_2 populations. This map spanned 1482rcM and contained 67 RFLP markers, 1078 AFLP markers obtained with 22 *Eco*RI + *Mse*I primer

combinations and 97 AFLP markers obtained with five *Pst*I + *Mse*I primer combinations, 231 AFLP markers being common to both populations.

The AFLP analysis method is more reproducible and robust than RAPD analysis and it displays more fragments than other fingerprinting techniques (Savelkoul *et al.* 1999). AFLP markers were also reported to be more efficient than RAPD markers to discriminate tomato lines though they did not reveal more polymorphism (Saliba-Columbani *et al.* 2000).

Smeich *et al.* (2000) reported the use of RAPD analysis using 271 primers to identify five primers, which enabled distinction of resistant and susceptible forms of tomato.

The use of randomly amplified polymorphic DNA to detect molecular markers linked to the tomato mosaic virus (ToMV) resistance gene '*Tm2nv*' in tomato has been reported by Tian *et al.* (2000).

Archak *et al.* (2002) reported low levels of polymorphism using RAPDs in tomato and difficulties for molecular chararacterization of cultivars in other diploid autogamous solanaceae species.

Gang *et al.* (2002) reported the use of RAPD marker analysis for identification of polymorphic markers for bacterial wilt resistance between resistant and susceptible bulk DNA of *Solanum phureja* using 300 random primers. The primer OPG09 gave a 960bp reproducible band in resistant clones in the population.

Balatero *et al.* (2002) reported high resolution detection using non-radioactive silver staining detection method for the construction of a molecular linkage map of F_6 recombinant inbred lines of tomato 'Hawaii 7996 x Wva 700' using polymerase chain reaction based markers such as amplified fragment length

polymorphism (AFLP), resistance gene analogues (RGA) and simple sequence repeats (SSR) for development of molecular markers for wilt resistance in tomato.

Tosti and Negri (2002) reported that though RAPD could efficiently discriminate among genetically distant accessions of cowpea, AFLP was more useful to analyze the diversity of cowpea populations that probably shared a more similar genetic pool.

Sharma (2003) reported that recent developments in molecular biology techniques particularly the advent of various DNA markers have greatly influenced plant protection methods. Various PCR-based and hybridization-based DNA marker techniques can be used for the chararacterization of genetic variability in pathogens and molecular tagging of disease resistance genes. DNA markers linked to specific resistance gene can be used in marker-assisted-selection for resistance breeding, gene pyramiding and map-based cloning of the resistance genes.

Chandrashekhara *et al.* (2003) reported high levels of genetic similarity between four species of tomato by the use of RAPD analysis using Operon primers belonging to the OPC, OPD, OPF, OPH and OPK series. The genetic dissimilarity coefficient between these species ranged from 0.16 to 0.40.

Menezes *et al.* (2003) from Brazil reported the presence of 21 AFLP primers revealing DNA bands unique to genotypes resistant to tomato spotted wilt virus and five primers revealing DNA bands associated with susceptibility from a total of 170 AFLP primer combinations surveyed for screening of tomato genotypes.

Chandrashekhara *et al.* (2003) reported the use of RAPD marker analysis to determine the extent of inter-specific genetic diversity in tomato. RAPD assay was carried out using 12 random decamer primers and four accessions

representing four species of tomato namely *L. esculentum*, *L. pimpinellifolium*, *L. glandulosum* and *L. hirsutum*.

Langella *et al.* (2004) reported the use of molecular marker-assisted breeding schemes using a CAPS marker derived from an RFLP marker CT220 for efficiently transferring TSWV resistance to two tomato elite lines AD-17 and Poly 39 from cultivar 'Stevens' carrying the *Sw-5–5* resistance gene in homozygous condition. They also highlight all the advantages of using molecular markers for selection.

Mejia *et al.* (2004) reported the absence of molecular marker for begomovirus resistance at resistance gene hotspots (Rgh) on chromosomes six and eleven of the tomato genome. They also identified tomato breeding lines Gh13, Gc 9 and Gc173 that are resistant to bipartite begomoviruses in Guatemala. Gh13 is the F_7 generation and is a homogenous breeding line with resistance derived from *Lycopersicon hirsutum.* Gc173 and Gc9 are F_8 breeding lines with resistance genes introgressed from *Lycopersicon chilense.*

The lack of polymorphism in RAPD analysis between eight *Solanum torvum* accessions and difficulties for molecular characterization of cultivars in other solanaceae species has been reported by Clain *et al.* (2004). They also reported that due to strong homologies between genomes of the solanaceae species, AFLPs may not be more polymorphic than RAPDs.

Sadashiva *et al.* (2006) identified 44 polymorphic SSR primers to distinguish between resistant and susceptible tomato plants out of a total of 94 SSR primers screened to identify polymorphism between ToLCV resistant parent IIHR-2101 and susceptible parent 15 SB SB.

Abraham *et al.* (2006) identified PCR-based marker TG105 to the *Ty2* gene conferring resistance to TYLCV in tomato, which could be used as a

molecular marker. They also reported the presence of an SSR marker to determine a tomato's susceptibility or resistance to ToLCV and aid in the creation of a commercially acceptable resistant hybrid.

Riccardia *et al.* (2007) reported that a recessive gene *Ol*-2 confers complete resistance to tomato powdery mildew, a new plant disease that in recent years has frequently occurred in open field and protected environments to cause serious damage to tomato crops. They have isolated eight new amplified fragment length polymorphism (AFLP) markers tightly linked to the *Ol*-2 gene for resistance, adding useful mapping information to the chromosome four region where *Ol*-2 locus is located.

Materials and Methods

3. MATERIALS AND METHODS

The study on molecular characterization of tomato genotypes for resistance to Tomato Leaf Curl Virus (ToLCV) was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and the Radio Tracer Laboratory, College of Horticulture, Vellanikkara during the period from 2005 to 2007. A description of the materials used and methodology adopted in the study has been furnished in this chapter.

3. 1 Collection of seed materials

Tomato germplasm available in AICVIP (All India Coordinated Vegetable Improvement Program), Vellanikkara were used for the present study. Seeds of the fifteen tomato genotypes selected for the study were obtained from different centers like the Asian Vegetable Research and Development Center (AVRDC), Taiwan; Himachal Pradesh Krishi Viswa Vidyalaya (HPKV), Palampur; Indian Institute of Vegetable Research (IIVR), Varanasi; Horticulture and Agroforestry Research Project (HARP), Ranchi; Orissa University of Agriculture and Technology (OUAT), Bhuvaneshwar and Kerala Agricultural University (KAU), Vellanikkara and maintained at the Department of Olericulture, Vellanikkara (Table1).

Table 1 Genotypes and their sources

Genotype	Source
Hawaii 7998, BL-333-3-1	HPKV, Palampur
LE-640, LE-638, LE-658, LE-651	AVRDC, Taiwan
H-24, H-86	IIVR, Varanasi
Swarna Lalima, Swarna Naveen	HARP, Ranchi
BT-218	OUAT, Bhuvaneshwar
Anagha, Sakthi, Mukthi, LE-474	KAU, Vellanikkara

a. Plants in nursery

b. One week after transplanting in pots

c. Performance of plants one month after transplanting

d. Plants after final harvest

Plate 1. Pot culture of selected tomato genotypes

3. 2 Evaluation of Tomato Genotypes for Resistance to ToLCV Disease

Seedlings of 15 genotypes were raised in sterilized nursery beds. Twenty eight-day-old seedlings were transplanted in pots and field for evaluating the genotypes for resistance/susceptibility to ToLCV disease.

3.2.1 Pot culture

Earthen pots were filled three-fourths with potting mixture containing soil, sand and cowdung (2:1:1). Potting mixture was sterilized using 40 per cent formaldehyde solution diluted @ 1:30. This solution was applied @ 1.5l / pot. Pots were then covered with polythene sheets. After a week, the sheets were removed and pots were then kept open for another week. Twenty eight-day-old seedlings were transplanted to these pots. All cultural practices followed were according to the Package of Practices of Recommendations: Crops (KAU, 2002).

Pots were arranged in Controlled Randomized Design containing two replications with each replication containing 10 plants per genotype (Plate 1). Genotypes were screened for resistance to ToLCV disease during the peak season for ToLCV infection (December – February). Reaction of genotypes to the disease was scored using 0 to 4 scale suggested by Banerjee and Kalloo (1998) (Table 2). Observations on disease incidence, symptomatology, stage of infection and occurrence of other diseases were recorded. Growth characters of the plants belonging to different genotypes were also recorded.

3.2.2 Field evaluation

Land was prepared by creating furrows 30 cm wide and 1.5 m long. After application and incorporation of manures and fertilizers according to the Package of Practices of Recommendations: Crops (KAU, 2002), the soil was sterilized using 40 per cent formaldehyde solution diluted @ 1:30. This solution was applied @ 4l/ furrow. After a week, twenty eight-day-old seedlings were

transplanted to the furrows at spacing of 60 cm between plants. The seedlings were given a temporary shade for two to four days.

Two replications with 10 plants in each replication were maintained for each genotype in a randomized block design (Plate 2). The genotypes were screened for resistance to ToLCV disease under natural conditions during the peak season of infection (December – February). The reaction of the genotypes to ToLCV disease was scored using the 0 to 4 scale suggested by Banerjee and Kalloo (1998) (Table 2). Observations on disease incidence, symptomatology, stage of infection and occurrence of other diseases were recorded. Biometric characters for all the plants of different genotypes were also recorded.

Per cent disease incidence, severity and coefficient of infection in pot culture and field experiment were calculated using the following formulae:

a. Per cent disease incidence $= \frac{\text{No: of infected plants}}{\text{Total no: of plants observed}} \times 100$

b. Per cent disease severity $= \frac{\text{Sum of numerical rating}}{\text{No: of plants observed x Maximum disease grade}} \times 100$

Per cent disease severity was calculated using the 0 to 4 score chart suggested by Banerjee and Kalloo (1998).

c. Coefficient of infection $= \frac{\text{Per cent disease incidence x Per cent disease severity}}{100}$

Based on the coefficient of infection genotypes were categorized into highly resistant, resistant, moderately resistant, moderately susceptible, susceptible and highly susceptible (Table 3).

Plate 2. Selected genotypes of tomato one week after transplanting in field

Table 2 Score chart of ToLCV disease severity

Disease Grade	Symptoms
0	Symptom absent
1	Very mild curling (up to 25% leaves)
2	Curling and puckering of 26-50% leaves
3	Curling and puckering of 51-75% leaves
4	Severe curling and puckering of >75% leaves

Table 3 Reaction of genotypes to ToLCV

Coefficient of Infection	Category
0-4	Highly resistant
4.1-9	Resistant
9.1-19	Moderately resistant
19.1-39	Moderately susceptible
39.1-69	Susceptible
69.1-100	Highly susceptible

The growth parameters observed in pot culture and field experiment were:

a. Plant height (cm)

   Height of each plant at 60 days after planting was recorded.

b. Internode length (cm)

   Internode length of each plant was recorded at 60 days after planting.

c. Number of primary branches per plant

   Number of primary branches in each plant was counted at 60 days after planting.

d. Average fruit weight (g)

Total weight of all fruits harvested from a single plant was estimated and divided by the total number of fruits.

e. Fruit size (cm)

Average fruit size for each genotype was recorded by calculating the mean of the equatorial and longitudinal diameters of 10 representative ripened fruits belonging to each genotype.

f. Yield per plant (g)

Yield of fruits from each plant was recorded.

Data collected on biometric characters for each genotype was statistically analyzed to determine significant differences if any among the characters.

3. 3 Artificial inoculation of ToLCV

Eight genotypes *viz.* Hawaii 7998, LE-658, LE-638, LE-651, LE-640, H-24, H-86 and Anagha, which were found highly resistant in pot and field studies were subjected to artificial inoculation using cleft grafting technique (Hill, 1984) to confirm the resistance of selected genotypes to ToLCV.

For virus transmission, only a short vascular contact is sufficient. In graft transmission, scions were excised from symptomatic parts of the ToLCV infected plant. The vascular system of the scions was exposed by two long cuts on the sides to create a wedge. In the recipient plant, leaves around the scion insertion site were removed, or the foliage was topped at the nodal region to prepare for graft transmission. A longitudinal cut was made in the stem into which the wedge-shaped scion was inserted. The graft insertion site was tied tightly with polythene tape and wrapped with moistened cotton. Grafted plants were covered with polythene cover to maintain the humidity. These covers were removed after two to three days. Observations on symptom development were recorded 10 to 30 days after grafting.

3. 4 Molecular Characterization

3.4.1 Chemicals, glassware and plastic ware used for the study

Chemicals used for the study were of good quality (AR grade) from various agencies such as MERCK, SRL and HIMEDIA. Molecular Biology Grade enzymes and buffers were supplied by Bangalore Genei Ltd. All the plastic ware used was obtained from Axygen and Tarson India Ltd. Primers for RAPD assay were obtained from Operon Technology, USA. γ^{32} P dATP was obtained from the Board of Radiation and Isotope Technology (BRIT), Mumbai. The list of instruments used in the study is given in Appendix I.

3.4.2 Standardization of Genomic DNA Isolation

Isolation of good quality genomic DNA from tomato is a prerequisite for RAPD and AFLP analysis. The procedures reported by Doyle and Doyle (1987) and Rogers and Bendich (1994) for the isolation of DNA were modified and tried for genomic DNA isolation from tomato. Isolation of DNA was done from tender leaves collected early in the morning.

3.4.2.1 DNA isolation by Doyle and Doyle Method

A modified protocol including modifications like the use of β-mercaptoethanol and sodium metabisulfite was followed to obtain DNA.

Reagents Used

1. Extraction buffer (4X)
2. Lysis buffer
3. TE buffer
4. Iso-propanol
5. Chloroform: Isoamyl alcohol mixture (24:1, v/v)

6. Sarcosin (5%)
7. Ethanol (100% and 70%)

Composition of respective reagents is provided in Appendix II.

Procedure

- Tender leaf tissue (1g) was ground in excess liquid nitrogen and the following reagents were added.
  4X Extraction buffer – 3 ml
  Sodium metabisulfite – 10 mg
  β-mercaptoethanol – 50 µl
- Homogenate was transferred into 50 ml Oakridge centrifuge tubes containing pre-warmed lysis buffer (4 ml) and added 1 ml of five per cent sarcosin.
- Mixture was maintained at 65°C for 10 to 15 minutes in a water bath with gentle mixing.
- Equal volume of chloroform: isoamyl alcohol mixture was added and mixed again.
- Homogenate was centrifuged at 10,000 rpm for 15 minutes at 4°C.
- The upper aqueous phase was saved and transferred to a fresh centrifuge tube after checking its volume. To this, 0.6 volume of chilled iso-propanol was added and mixed gently and then incubated at -20°C for 30 minutes for precipitation of DNA.
- DNA was pelleted by centrifugation at 10,000 rpm for 15 minutes at 4°C.
- Supernatant was discarded and the pellet was washed with 70 per cent ethanol followed by wash with absolute alcohol.
  The pellet was air dried for 30 minutes, dissolved in 250 µl TE buffer and stored at -20°C.

24

3.4.2.2 DNA Isolation Method by Rogers and Bendich

The original protocol along with modifications like addition of β-mercaptoethanol and changing the quantity of extraction buffer was followed.

Reagents Used

1. 2X CTAB extraction buffer
2. 10% CTAB solution
3. TE buffer
4. Iso-propanol
5. Chloroform: Isoamyl alcohol mixture (24:1, v/v)
6. Ethanol 100% and 70%

Composition of reagents is provided in Appendix II.

Procedure

- Tender leaf tissue (1g) was ground in excess liquid nitrogen and 4 ml of 2X extraction buffer and 100 µl β-mercaptoethanol were added.
- The ground tissue was transferred in to a 50 ml Oakridge tube containing 3 ml pre-warmed extraction buffer. The contents were mixed well and incubated at 65°C for 15 minutes.
- Equal volume (7 ml) of chloroform: isoamyl alcohol mixture was added, mixed gently by inversion and centrifuged at 10,000 rpm for 10 minutes at 4°C.
- Upper aqueous phase containing DNA was transferred to a fresh Oakridge tube and $1/10^{th}$ of its volume of 10 per cent CTAB solution was added and mixed gently by inversion.
- Equal volume of chloroform: isoamyl alcohol mixture was added, mixed gently to form an emulsion and centrifuged at 10,000 rpm for 10 minutes at 4°C.

- Upper aqueous phase was collected in a fresh oakridge tube and 0.6 its volume of chilled iso-propanol was added and mixed gently to precipitate the DNA. It was incubated at -20°C for 30 minutes.
- The contents were centrifuged at 10,000 rpm for 5 minutes at 4°C to pellet the DNA.
- Supernatant was discarded and the pellet was washed with 70 per cent ethanol followed by absolute alcohol.
- The pellet was air dried for 30 minutes, dissolved in 250 µl TE buffer and stored at -20°C.

3.4.2.3 Purification of DNA

Isolated DNA contained RNA as contaminant. This was purified by phenol precipitation and RNase treatment (Sambrook *et al.*, 1989).

Reagents Used

1. Phenol: Chloroform mixture (1:1, v/v)
2. Chilled iso-propanol
3. Ethanol (70%)
4. TE buffer
5. Chloroform: Isoamyl alcohol mixture (24:1, v/v)
6. RNase

RNase from Sigma, USA was used to prepare RNase. One per cent solution was prepared by dissolving RNase A in TE buffer at 100°C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20°C.

Procedure followed for DNA purification was as follows:

- RNase solution (2 µl) was added to 100 µl DNA sample and incubated at 37°C in a dry bath (Genie, Thermocon) for one hour.

- Volume was made up to 250 µl with distilled water and equal volume of phenol: chloroform mixture was added.
- Mixture was centrifuged at 12,000 g for 10 minutes at 4°C.
- The aqueous phase was collected in a fresh microfuge tube and equal volume of chloroform: isoamyl alcohol mixture was added.
- This solution was centrifuged at 10,000 rpm for 10 minutes at 4°C.
- Above two steps were repeated and finally the DNA was precipitated from the aqueous phase with 0.6 volume of chilled iso-propanol.
- Mixture was incubated at -20°C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4°C.
- DNA pellet was washed with 70 per cent ethanol.
- Pellet was air dried and dissolved in 25 µl TE buffer and stored.

3.4.2.4 Estimation of Quality of DNA by Agarose Gel Electrophoresis

Agarose gel electrophoresis (Sambrook *et al.*, 1989) was carried out to determine the quality of the isolated DNA.

Reagents Used

1. Agarose (0.8%)
2. TAE Buffer 50X (pH 8)
3. Loading dye (6X)
4. Intercalating dye (Ethidium Bromide)

Composition of the reagents is given in Appendix III.

Procedure

TAE buffer (50X) was diluted to 1X concentration. Required quantity of agarose was weighed and added to the required volume of 1X TAE buffer to make 0.8 per cent solution. The mixture was heated to melt the agarose. The solution

containing the melted agarose was allowed to cool (40°C) and then ethidium bromide (0.5 µg/ ml) was added. The solution was mixed well and poured into a gel-casting tray whose open ends were sealed with cello tape. The tray was placed on a level surface and an appropriate comb was properly placed before setting the gel. After solidification of the gel, the comb and the cello tape were carefully removed.

The gel-casting tray containing the gel was placed in the electrophoresis tank (Biorad) containing 1X TAE buffer. The gel was placed such that the wells in the gel were on the cathode side of the electrophoresis tank and was completely immersed in the buffer.

The DNA samples (3 µl) were mixed with loading dye (1 µl) and carefully loaded into the wells. Molecular weight marker (λ DNA/ *Eco*RI + *Hind*III double digest, Bangalore Genei) was also loaded in one well. After the gel tank was closed, the cathode and anode were connected to the power pack and current was passed at constant voltage (100V) till the loading dye reached two-third the length of the gel.

3.4.2.5 Gel Documentation

The gel was removed from the electrophoresis unit and placed over the UV transilluminator in the gel documentation system (Alpha Imager TM 1200). The DNA bands were visualized and documented in the computer. Quality of the DNA extracted was ensured.

3.4.2.6 Spectrophotometric Analysis of DNA

The quantity and quality of DNA was evaluated by spectrophotometry. Two micro litre of DNA was diluted to 1.5 ml with distilled water. Absorbance at wavelengths 260nm and 280nm were taken using the Spectronic R Genesys 5 spectrophotometer. Distilled water was used as blank. Purity of DNA was

assessed from the ratio of absorbance at 260nm and 280nm. A value of 1.8 indicates good quality DNA.

DNA in the good quality sample was quantified by the equation,

Absorbance at 260nm = 1 is equivalent to 50 µg double stranded DNA per ml.

Therefore, absorbance at 260nm x 50 gives the quantity of DNA in µg per ml.

3.4.3 RAPD Analysis

After the isolation and quantification of good quality DNA from the tomato genotypes, RAPD analysis was carried out using the isolated DNA as template.

RAPD (Random Amplified Polymorphic DNA) analysis is a PCR-based molecular marker technique that uses arbitrarily selected single, short oligonucleotide primers to amplify a set of DNA fragments distributed randomly throughout the genome. Decamer primers are normally used. Use of decamer primers gives a number of amplification products from random locations in the genome that can be separated using a 1.2 per cent agarose gel. The differences between individuals can be observed as different banding patterns on the gel.

An RAPD reaction mixture contains different constituents like template DNA, random primer, Taq DNA polymerase enzyme, dNTPs, magnesium chloride and assay buffer, which are subjected to repeated cycles of denaturation, primer annealing and extension in a thermal cycler. The different cycles followed in an RAPD reaction are as follows:

Initial denaturation : 94°C for 5 minutes

Denaturation	: 94°C for 1 minute	40 cycles
Primer annealing	: 37°C for 1 minute	
Primer extension	: 72°C for 2 minutes	

Final extension : 72°C for 5 minutes

Holding temperature: 4°C for infinity

29

3.4.3.1 Primer Screening and RAPD Analysis

Primer screening was carried out to identify the best primers for RAPD analysis. Twenty decamer primers each, belonging to the OPS and OPY series obtained from Operon Technologies were screened (Table 4 and 5).

Genomic DNA belonging to a single genotype was used as template for all reactions during screening. A master mix containing all components except the primer was prepared. Components of the master mix and quantity of DNA for each reaction is given in Table 6. The reactions were set up in 0.2 ml microfuge tubes placed on ice by pipetting out the master mix as aliquots. Then the different primers were added separately to the different tubes. The tubes were subjected to a short spin and then were placed in a thermal cycler and the reaction was carried out using the above-mentioned RAPD program. The products were checked on a 1.2 per cent agarose gel stained with ethidium bromide and then documented.

After initial primer screening, primers showing good amplification were selected and further RAPD analysis of the 15 tomato genotypes was carried out. Primers showing polymorphism in the banding pattern among genotypes were selected and RAPD assay using these primers was repeated for confirmation of polymorphism.

3.4.3.2 Analysis of Banding Patterns in RAPD

Amplification profiles of the 15 genotypes were compared with each other. Bands were scored manually as 1 or 0 depending on their presence or absence. The data was analyzed using Numerical Taxonomy System of Multivariate Statistical Program (NTSyS) software package (Rohlf, 1990). The SIMQUAL program was used to calculate Jaccard's cofficient, a common estimator of genetic identity. Clustering was done using Sequential Agglomerative Hierarchical Nested Clustering (SAHN) and a dendrogram was constructed using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) (Sneath and Sokal, 1973) using the NTSyS package.

30

Table 4 Nucleotide Sequence of OPS Primer Series

Sl. No	Primer	Nucleotide Sequence
1	OPS 1	GTTTCGCTCC
2	OPS 2	TGATCCCTGG
3	OPS 3	CATCCCCCTG
4	OPS 4	GGACTGGAGT
5	OPS 5	TCGGCCCTTC
6	OPS 6	TGCTCTGCCC
7	OPS 7	GGTGACGCAG
8	OPS 8	GTCCACACGG
9	OPS 9	TGGGGGACTC
10	OPS 10	CTGCTGGGAC
11	OPS 11	GTAGACCCGT
12	OPS 12	CCTTGACGCA
13	OPS 13	TTCCCCCGCT
14	OPS 14	TCCGCTCTGG
15	OPS 15	GGAGGGTGTT
16	OPS 16	TTTGCCCGGA
17	OPS 17	AGGGAACGAG
18	OPS 18	CCACAGCAGT
19	OPS 19	ACCCCCGAAG
20	OPS 20	GGACCCTTAC

Table 5 Nucleotide Sequence of OPY Primer Series

Sl. No	Primer	Nucleotide Sequence
1	OPY 1	GTGGCATCTC
2	OPY 2	CATCGCCGCA
3	OPY 3	ACAGCCTGCT
4	OPY 4	GGCTGCAATG
5	OPY 5	GGCTGCGACA
6	OPY 6	AAGGCTCACC
7	OPY 7	AGAGCCGTCA
8	OPY 8	AGGCAGAGCA
9	OPY 9	AGCAGCGCAC
10	OPY 10	CAAACGTGGG
11	OPY 11	AGACGATGGG
12	OPY 12	AAGCTTGCGA
13	OPY 13	GGGTCTCGGT
14	OPY 14	GGTCGATCTG
15	OPY 15	AGTCGCCCTT
16	OPY 16	GGGCCAATGT
17	OPY 17	GACGTGGTGA
18	OPY 18	GTGGAGTCAG
19	OPY 19	TGAGGGTCCC
20	OPY 20	AGCCGTGGAA

Table 6 Components of RAPD reaction

Components	Concentration	Quantity for 20µl
Taq DNA Polymerase buffer (10X)	1X	2 µl
dNTP mix	10mM each	1 µl
Magnesium Chloride	1.25mM	1 µl
Random primer	10 picomoles (0.5µM)	1.5 µl
Taq DNA Polymerase	1 U/µl	0.3 µl
Template DNA	25ng	2 µl
Sterile Water	To make up to 20µl	12.2 µl

Resolving power (Prevost and Wilkinson, 1999) was used to identify primers that would distinguish between accessions more efficiently. Resolving power (Rp) of a primer is calculated as the sum of 'band informativeness' of all the bands produced by the primer. Band informativeness (Ib) = 1 - (2 x |0.5-p|), where 'p' is the proportion of accessions containing the band. Resolving power of the primer is represented as: Rp= Σlb.

3.4.4 AFLP Analysis

AFLP (Amplified Fragment Length Polymorphism) analysis is a highly sensitive method for detecting polymorphisms throughout the genome. It is a highly reproducible analysis. It is based on the selective amplification of a subset of genomic restriction fragments using polymerase chain reaction.

AFLP involves restriction endonuclease digestion of the DNA and ligation of adaptors, amplification of the restriction fragments and gel analysis of amplified fragments. Different combinations of *Eco*RI and *Mse*I based primer pairs are used for different AFLP reactions. AFLP analysis allows co-amplification of 50 to 100 restriction fragments in a single reaction. Denaturing polyacrylamide gels are used for analysis. The resultant banding pattern can be documented and analyzed either manually or by the use of analytical software to detect polymorphisms.

3.4.4.1 Protocol

AFLP was carried out using AFLP® Analysis System I kit obtained from Invitrogen Corporation, USA. Composition of different reagents used in AFLP analysis is given in Appendix IV. The method involves the following steps:

3.4.4.1.1 Restriction digestion of genomic DNA

The following components involved were added to a 1.5 ml microfuge tube.

Component	Quantity/ sample
5X Reaction buffer	5 µl
Genomic DNA	2 µl
*Eco*RI/*Mse*I	2 µl
Distilled water	16 µl
Total volume	25 µl

The contents of the tubes were mixed gently and centrifuged briefly. The mixture was incubated for two hours at 37°C. After this, the contents were incubated again at 70°C for 15 minutes to inactivate restriction enzymes. Tubes were then placed on ice and centrifuged briefly.

3.4.4.1.2. Adapter ligation

Adapter/ligation solution (24 µl) and T4 DNA ligase (1 µl) were added to the digested DNA. The contents of the tubes were mixed gently and centrifuged briefly. The tubes were then incubated at 20±2°C for two hours.

Ligated mixture was diluted 10 times using TE buffer. For this 90 µl TE buffer was added to 10 µl of the reaction mixture and mixed well. Unused portion of the reaction mixture was stored at -20°C.

3.4.4.1.3 Pre-amplification reactions

Components for pre-amplification were added to a 0.2 ml microfuge tube.

Component	Volume
Dilute ligated template DNA	5 µl

Pre-amp primer mix	40 µl
10X PCR buffer plus Mg	5 µl
Taq DNA polymerase (5U/µl)	1 µl
Total volume	51 µl

The contents were mixed gently and centrifuged briefly to collect the reaction. The tubes were placed in a thermal cycler and the PCR program consisting of 20 cycles for pre-amplification was set.

Pre-amplification Program
94°C for 30s
56°C for 60s
72°C for 60s
Holding temperature = 4°C

Pre-amplified samples were diluted 50 times with TE buffer by adding 147 µl TE buffer to 3 µl of pre-amplified sample. Diluted and undiluted samples were stored at -20°C.

3.4.4.1.4 Primer labeling

Primer labeling was performed by phosphorylating the 5` end of the *Eco*RI primers with γ^{32}P ATP and T4 kinase.

The following components were added to a 1.5 ml microfuge tube for labeling of *Eco*RI primer.

Component	**Volume (for 10 samples)**
*Eco*RI primer	1.8 µl
5X kinase buffer	1 µl
γ^{32}P ATP	2 µl
T4 kinase	0.2 µl
Total volume	5µl

36

The contents were gently mixed and centrifuged briefly. The reaction mixture was incubated at 37°C for one hour. This was followed by heat inactivation of the enzyme at 70°C for 10 minutes.

Simultaneously, the 30-330bp AFLP ladder (Invitrogen) was also labeled using γ^{32} P dATP. The reaction was set up as follows.

Component	Volume
30-330bp AFLP ladder	2 µl
5X Exchange reaction buffer	1 µl
γ^{32}P dATP	1 µl
T4 polynucleotide kinase	1 µl
Total Volume	5 µl

The components were mixed thoroughly, collected by brief centrifugation and incubated for 10 minutes at 37°C. Then the reaction was inactivated at 65°C for 15 minutes. An equal volume (5 µl) of TE buffer was added to the reaction mixture followed by 20 µl of denaturing solution. The solution was incubated at 70°C for five minutes and stored at -20°C.

3.4.4.1.5 Selective amplification

For each primer pair, the following components were added to a 1.5 ml microfuge tube and the tube was labeled 'Mix 1'.

Component	Volume
Labeled *Eco*RI primer	5 µl
Mse I primer mix (contains dNTPs)	45 µl
Total volume	50 µl

Mix 2 – The following components were added to another 1.5 ml microfuge tube to get 'Mix 2'.

Component	Volume
10X PCR buffer plus Mg	20 µl
Taq DNA polymerase (5U/µl)	1 µl
Distilled water	79 µl
Total volume	100 µl

AFLP reaction mixture (total volume 20 µl) was prepared by adding 5 µl of pre-amplified diluted template DNA, 5 µl of 'Mix 1' and 10 µl 'Mix 2' into a 0.2 ml microfuge tube. The contents were gently mixed and centrifuged briefly and placed in a thermal cycler for selective amplification with the following conditions: one cycle at 94°C for 1 minute, 65°C for 1 minute and 72°C for 1 hour 30 minutes. Next, the annealing temperature was lowered in each cycle by 0.7°C during 12 cycles. This was followed by 23 cycles at 94°C for 30 seconds, and 72°C for 1 minute. After completion of amplification, the reaction was held at 4°C.

3.4.4.1.6 Poly Acrylamide Gel Electrophoresis (PAGE)

The AFLP products were separated on a four per cent denaturing poly acrylamide gel for viewing the AFLP banding pattern. The steps involved in PAGE are as follows:

A. Preparation of 4% acrylamide solution

Forty per cent acrylamide-bis acrylamide (19:1) solution was first prepared by dissolving 38 g of acryalmide and 2 g of bis acrylamide in 100 ml of double distilled water. Urea (420 g) was dissolved in approximately 400 ml of warmed (50°C) double-distilled water and 100 ml of 5X TBE was added to this solution. The solutions were mixed well and volume was made up to 1000 ml by adding double distilled water. The resulting solution (4% working solution) was filtered and stored in a brown bottle for further use.

B. Casting the gel

The gel casting unit consists of two glass plates separated by spacers and a shark's-tooth comb. The glass plates, spacers and comb were cleaned well first with distilled water and then with cent per cent ethanol. The plates were then allowed to dry. Repel silane (50 µl) was evenly spread using a tissue paper on the thermostatic plate. Spacers were placed on the sides of this plate. The other glass plate was placed evenly on the thermostatic plate such that both plates were separated by spacers. This gel-casting unit was then fitted by tightening clamps on both sides of the plates, fitted with a support and kept horizontally such that the second glass plate faced downwards.

The shark's-tooth comb was inserted at the top of the gel-casting unit with the even edge facing the gel. Ten per cent APS (800 µl) and 80 µl TEMED were added to 80 ml of four per cent acrylamide working solution taken in a 100 ml beaker and mixed well. The prepared gel was immediately injected into the gel-casting unit through the bottom side of the unit using a syringe till the gel reached the top of the plate. Injection was done speedily to avoid formation of air bubbles. The plate assembly was kept undisturbed for 30 minutes to allow polymerization of the gel.

C. Pre-running

After polymerization of the gel, the gel casting assembly was placed vertically in the electrophoresis tank containing 0.5X TBE buffer. The cavity in the thermostatic plate was also filled to the brim with 0.5X TBE buffer. The assembly was connected to the power pack and subjected to pre-running to achieve a temperature of 50°C at 40W power.

D. Sample loading

Equal volume of formamide dye (20 µl) was added to the amplified product to obtain a dye concentration of 1X in the reaction mixture. Samples and the molecular weight ladder marker were mixed well and denatured for five minutes at 94°C in a thermal cycler.

After the pre-run, the shark's-tooth comb was removed and top surface of the gel was cleaned. The shark's-tooth comb was re-inserted into the gel surface with the even side up to create wells. Eight micro litre of denatured sample was loaded into each well. Denatured labeled molecular weight ladder marker (pUC 19) (2 µl) was also loaded in one well. Current was passed through the gel for 90 to 120 minutes at 40W power and temperature range of 45°C to 50°C.

E. Gel drying

After electrophoresis, the buffer was poured off, gel clamps were removed and the plates were cooled to room temperature. The comb was removed and then plates were separated. The gel attached to the smaller glass plate was detached from it by blotting it on to a filter paper placed over it. A cling film was used to cover the gel and the gel was then dried in a gel dryer (Biorad) for two hours at 80°C.

F. Visualization of AFLP banding patterns

Dried gel was placed in cassette and exposed to a BAS-IP MS FUJIFILM imaging plate for 15 minutes. The image was then viewed in a Phosphor Imager (FLA-5100, Fuji) and documented using the Fluorescent Image Analyzing system software.

3.4.4.2 Gel analysis

The AFLP amplification profiles for the tomato genotypes were compared with each other and scored as 0 and 1 for the absence and presence of bands. Scored data was analyzed using the NTSyS (Numerical Taxonomy System of Multivariate Statistical Program) software. The SIMQUAL program was used to create a pair-wise similarity matrix.

Clustering was done using Sequential Agglomerative Hierarchical Nested Clustering (SAHN) and a dendrogram was constructed using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) (Sneath and Sokal, 1973). The relationships among the genotypes selected for the study were analyzed based on the similarity matrix and the dendrogram obtained.

Results

4. RESULTS

Results obtained from the research on molecular characterization of tomato genotypes for resistance to ToLCV are described in this chapter.

4.1 Reaction of Tomato Genotypes to ToLCV Disease

4.1.1 Screening under pot culture conditions

Seedlings belonging to the 15 genotypes transplanted and raised in earthen pots that contained sterilized potting mixture recorded good initial growth and the symptoms of leaf curl disease were observed mainly during the later stages of plant growth (Plate 3). Individual plants belonging to the different genotypes were scored for their reaction to ToLCV disease using the 0 to 4 score chart of Banerjee and Kalloo (1998). Per cent ToLCV disease incidence and severity recorded and coefficient of infection (CI) calculated for each genotype is presented in Table 7.

From the table it is observed that among the 15 genotypes tested, nine were highly resistant to ToLCV of which six genotypes namely, Hawaii 7998, H-24, H-86, LE-658, LE-474 and LE-640 were completely free of ToLCV incidence and three genotypes LE-638, LE-651 and Anagha recorded CI values of 1.0 to 4.0.

In Swarna Lalima, Swarna Naveen and Sakthi coefficient of infection ranged from 42.5 to 65.3 and these were categorized as susceptible. The genotypes BT-218 (82.5), BL-333-3-1 (76.6) and Mukthi (77.5) were found to be highly susceptible to virus infection.

4.1.2 Screening under field conditions

Fifteen tomato genotypes raised in sterile soil in the field, screened for resistance to ToLCV disease during the period from December 2006 to February 2007, the peak season for ToLCV incidence in Kerala and scored using 0 to 4

Plate 3. ToLCV infection in pot culture

Table 7 Reaction of tomato genotypes to ToLCV under pot culture conditions

Sl. No	Genotypes	Per cent Disease Incidence	Per cent Disease Severity	Coefficient of Infection	Disease reaction
1	Hawaii 7998	0	0	0	Highly resistant
2	H-24	0	0	0	Highly resistant
3	H-86	0	0	0	Highly resistant
4	LE-658	0	0	0	Highly resistant
5	LE-638	10	10	1.0	Highly resistant
6	LE-640	0	0	0	Highly resistant
7	LE-651	20	20	4	Highly resistant
8	LE-474	0	0	0	Highly resistant
9	BL-333-3-1	100	76.6	76.6	Highly susceptible
10	BT-218	100	82.5	82.5	Highly susceptible
11	Swarna Lalima	90	72.5	65.3	Susceptible
12	Swarna Naveen	100	42.5	42.5	Susceptible
13	Anagha	10	10	1.0	Highly resistant
14	Sakthi	70	63.3	44.3	Susceptible
15	Mukthi	100	77.5	77.5	Highly susceptible

43

scale for their reaction to ToLCV differed in their disease reaction (Plate 4) and were grouped into different categories based on the coefficient of infection. The details are provided in Table 8.

According to the data obtained, the genotypes Hawaii 7998, H-24, H-86, Anagha, LE-638, LE-658, LE-651 and LE-640 were found to be highly resistant to tomato leaf curl disease and disease incidence was not noticed in these genotypes. LE-474 with CI value (4.6) lying between 4.1 and 9 was found to be resistant to infection. Genotype BL-333-3-1 with CI value of 34 was categorized as moderately susceptible to infection, while other genotypes were susceptible (Swarna Naveen and Sakthi) and highly susceptible (BT-218, Swarna Lalima and Mukthi) to the disease.

4.1.3 Symptomatology and Stage of Infection

Symptoms of the disease included upward curling of leaves, cupping of leaves, appearance of violet tinge on the curled leaves and yellowing of leaves as shown in Plate 5. The disease was observed in the later stages of crop growth. Symptoms were not observed during the seedling stage of the crop.

4.1.4 Confirmation of resistance to ToLCV by artificial inoculation

Selected genotypes Hawaii 7998, H-24, H-86, Anagha, LE-638, LE-658, LE-651 and LE-640 observed to be resistant to ToLCV disease were artificially inoculated by graft transmission to confirm the resistance to ToLCV infection with Mukthi as susceptible check. The selected genotypes were grafted with diseased scions. Cleft grafting technique was adopted for graft inoculation (Plate 6). Observations were recorded from 10 days after grafting.

The newly emerging leaves in Mukthi showed typical curling symptoms within 10 days after grafting, while newly emerged leaves of resistant genotypes

Highly resistant genotype - Hawaii 7998

Moderately susceptible
genotype - BL-333-3-1

Highly susceptible
genotype - Swarna Lalima

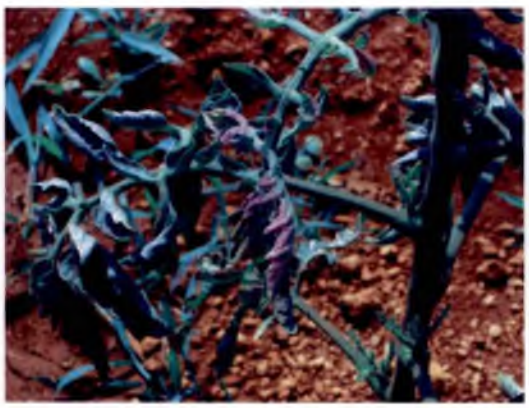
Plate 4. Difference in ToLCV reaction in field

Table 8 Reaction of tomato genotypes to ToLCV disease under field condition

Sl. No	Genotypes	Per cent Disease Incidence	Per cent Disease Severity	Coefficient of Infection	Disease reaction
1	Hawaii 7998	0	0	0	Highly resistant
2	H-24	0	0	0	Highly resistant
3	H-86	0	0	0	Highly resistant
4	LE-658	0	0	0	Highly resistant
5	LE-638	0	0	0	Highly resistant
6	LE-640	0	0	0	Highly resistant
7	LE-651	0	0	0	Highly resistant
8	LE-474	27.7	16.6	4.6	Resistant
9	BL-333-3-1	80	42.5	34	Moderately susceptible
10	BT-218	100	83.3	83.3	Highly susceptible
11	Swarna Lalima	100	77.7	77.7	Highly susceptible
12	Swarna Naveen	85.7	47.6	40.8	Susceptible
13	Anagha	0	0	0	Highly resistant
14	Sakthi	100	61.1	61.1	Susceptible
15	Mukthi	100	83.8	83.8	Highly susceptible

Severe curling

Cupping of leaves



Curling with violet tinge

Curling and yellowing

Plate 5. Symptoms of ToLCV disease

a. Resistant genotypes maintained after graft inoculation

b. Emergence of healthy shoots after graft inoculation

Plate 6. Artificial inoculation by cleft grafting

did not show any symptoms of viral infection till 30 days after grafting, which confirmed the resistance of these genotypes to ToLCV disease.

4.1.5 Comparison of Genotypes Based on Biometric Characters

The results of the comparison of genotypes based on growth parameters like plant height, internode length, number of primary branches, average fruit weight, fruit size and per plant yield under field and pot culture conditions are summarized in Tables 9 and 10.

A. Pot Culture Experiment

Statistical analysis of the data on plant height, internode length, number of primary branches, average fruit weight, fruit size and per plant yield under pot culture conditions showed significant differences among genotypes for each of the characters mentioned (Table 9).

Plant height observed ranged from 53.75cm to 102.2cm with the genotype H-86 showing the minimum (53.75cm) and the genotype LE-640 showing the maximum plant height (102.2cm). Other genotypes showing plant height comparable with that of LE-640 were LE-651 (83.2cm) and Swarna Naveen (92.2cm).

Maximum internode length was observed in case of LE-638 (6.5cm) and minimum in H-86 (4.13cm). Other genotypes with internode length statistically on par with the genotype LE-638 were LE-640 (5.95cm), LE-651 (5.6cm) and BT-218 (5.75cm).

Number of primary branches observed in the genotypes ranged from two to four with the genotype Mukthi having the highest number of primary branches. The genotypes H-24 and H-86 were found to be statistically on par with Mukthi

Table 9 Biometric characters of tomato genotypes under pot culture experiment

Sl. No	Genotypes	Plant Height (cm)	Internode length (cm)	No: of primary branches	Average fruit weight (g)	Yield/plant (g)	Fruit size (cm)
1	Hawaii7998	60.40 ^{cd}	4.70 ^{def}	2.80 ^{b}	23.96 ^{def}	220.6 ^{g}	3.29 ^{cd}
2	H-24	55.60 ^{d}	4.38 ^{ef}	3.60 ^{a}	19.98 ^{efg}	351.8 ^{b}	3.33 ^{bcd}
3	LE-474	63.00 ^{cd}	4.89 ^{cdef}	2.40 ^{b}	16.93 ^{g}	325.5 ^{bcd}	3.16 ^{d}
4	LE-658	76.95 ^{bcd}	5.13 ^{bcde}	2.10 ^{b}	46.43 ^{a}	215.8 ^{g}	4.08 ^{a}
5	LE-638	64.70 ^{cd}	6.50 ^{a}	2.10 ^{b}	29.03 ^{cd}	239.4 ^{fg}	3.70 ^{abc}
6	LE-640	102.2 ^{a}	5.95 ^{ab}	2.00 ^{b}	29.81 ^{cd}	270.2 ^{ef}	3.39 ^{bcd}
7	LE-651	83.20 ^{abc}	5.60 ^{abcd}	2.70 ^{b}	19.60 ^{fg}	301.3 ^{cde}	3.33 ^{bcd}
8	H-86	53.75 ^{d}	4.13 ^{f}	3.75 ^{a}	36.22 ^{b}	169.0 ^{h}	3.29 ^{cd}
9	Anagha	60.30 ^{cd}	4.95 ^{bcdef}	2.50 ^{b}	21.02 ^{efg}	282.4 ^{def}	3.36 ^{bcd}
10	BL-333-3-1	68.20 ^{bcd}	4.95 ^{bcdef}	2.80 ^{b}	25.81 ^{de}	275.4 ^{ef}	3.21 ^{d}
11	BT-218	75.15 ^{bcd}	5.75 ^{abc}	2.60 ^{b}	33.54 ^{bc}	157.2 ^{h}	3.74 ^{ab}
12	Swarna Lalima	72.90 ^{bcd}	5.37 ^{bcde}	2.30 ^{b}	34.06 ^{bc}	415.9 ^{a}	3.18 ^{d}
13	Swarna Naveen	92.20 ^{ab}	5.40 ^{bcd}	2.70 ^{b}	18.46 ^{fg}	298.2 ^{cde}	3.28 ^{cd}
14	Sakthi	61.20 ^{cd}	5.13 ^{bcde}	2.50 ^{b}	18.38 ^{fg}	304.3 ^{cde}	3.19 ^{d}
15	Mukthi	56.60 ^{d}	5.09 ^{bcdef}	4.05 ^{a}	21.26 ^{efg}	342.4 ^{bc}	2.99 ^{d}

All values are mean of two replications
In each column figures followed by same letter don't differ significantly accoding to DMRT

Table 10 Biometric characters of tomato genotypes under field condition

Sl. No	Genotypes	Plant Height (cm)	Internode length (cm)	No: of primary branches	Average fruit weight (g)	Yield/plant (g)	Fruit size (cm)
1	Hawaii7998	53.55 ^{b}	5.25 ^{b}	2.00 ^{de}	26.15 ^{de}	274.5 ^{e}	3.25 ^{d}
2	H-24	33.30 ^{f}	4.25 ^{c}	2.00 ^{de}	20.00 ^{f}	135.0 ^{gh}	2.60 ^{e}
3	LE-474	37.45 ^{def}	3.75 ^{cd}	2.15 ^{de}	25.75 ^{de}	420.0 ^{c}	3.15 ^{d}
4	LE-658	51.22 ^{b}	5.65 ^{b}	3.60 ^{a}	44.60 ^{a}	534.5 ^{b}	4.60 ^{a}
5	LE-638	37.90 ^{def}	3.75 ^{cd}	2.00 ^{de}	37.75 ^{b}	436.0 ^{c}	3.95 ^{b}
6	LE-640	66.70 ^{a}	7.05 ^{a}	1.75 ^{e}	36.50 ^{bc}	362.5 ^{d}	3.95 ^{b}
7	LE-651	45.80 ^{c}	3.90 ^{cd}	2.80 ^{bcd}	21.50 ^{ef}	120.0 ^{h}	3.05 ^{d}
8	H-86	40.45 ^{d}	3.80 ^{cd}	2.00 ^{de}	32.55 ^{c}	166.0 ^{g}	3.25 ^{d}
9	Anagha	36.65 ^{def}	4.40 ^{c}	2.40 ^{cde}	24.00 ^{def}	323.7 ^{d}	3.10 ^{d}
10	BL-333-3-1	42.15 ^{cd}	3.35 ^{d}	2.40 ^{cde}	24.05 ^{def}	260.8 ^{e}	3.20 ^{d}
11	BT-218	38.55 ^{def}	3.40 ^{d}	3.00 ^{abc}	25.85 ^{de}	342.3 ^{d}	3.55 ^{c}
12	Swarna Lalima	39.00 ^{de}	4.15 ^{c}	1.75 ^{e}	36.25 ^{bc}	214.4 ^{f}	3.65 ^{c}
13	Swarna Naveen	42.00 ^{cd}	4.25 ^{c}	2.00 ^{de}	20.85 ^{f}	105.4 ^{h}	2.55 ^{e}
14	Sakthi	40.45 ^{d}	3.85 ^{cd}	2.50 ^{cde}	33.60 ^{bc}	641.5 ^{a}	4.00 ^{b}
15	Mukthi	33.50 ^{ef}	3.40 ^{d}	3.30 ^{ab}	27.31 ^{d}	361.0 ^{d}	2.70 ^{e}

All values are mean of two replications

In each column figures followed by same letter don't differ significantly accoding to DMRT

with respect to the number of primary branches. All other genotypes were found to show significant difference from the genotypes Mukthi, H-24 and H-86 with respect to number of primary branches.

Maximum average fruit weight and fruit size was observed in genotype LE-658 with average fruit weight of 46.43 g and fruit size of 4.08cm as shown in Plate 7. Minimum average fruit weight was observed in genotype LE-474 (16.93 g) and smallest fruits were observed in genotype Mukthi with fruits of size 2.99cm (Plate 8). The genotypes LE-638 with 3.7cm and BT-218 with 3.74cm respectively were statistically similar to the genotype LE-658 with respect to fruit size. All genotypes showed significant differences in average fruit weight.

Per plant yield in pot culture ranged from 157.2 g to 415.9 g with the genotype BT-218 showing the lowest and Swarna Lalima displaying the highest values. Swarna Lalima was significantly superior to all other genotypes with respect to the per plant yield (Plate 9).

B. Evaluation Under Field Conditions

The genotypes displayed significant differences on statistical analysis of data collected for characters such as plant height, internode length, number of primary branches, average fruit weight, fruit size and per plant yield under field conditions (Table 10).

Plant height under field conditions ranged from 33.3cm to 66.7cm. The genotypes H-24 and LE-640 showed the minimum and maximum height. All genotypes differed significantly from the maximum value (LE-640) with respect to plant height.

Plants belonging to the 15 genotypes displayed a wide variation in the length of internodes. Analysis of data obtained on internode length of genotypes

Plate 7. Resistant genotype LE-658 with maximum fruit size

Plate 8. Susceptible genotype Mukthi with minimum fruit size in pot culture

Plate 9. Susceptible genotypes Sakthi and Swarna Lalima with maximum per plant yield

revealed that maximum internode length was shown by genotype LE-640 with a value of 7.05cm. Minimum internode length was observed in the genotype BL-333-3-1 (3.35cm).

Number of primary branches observed in the genotypes ranged from approximately one to four with the least in LE-640 (1.75) and Swarna Lalima (1.75) and maximum in LE-658 (3.6).

Genotype LE-658 was found to be significantly superior to all other genotypes with respect to average fruit weight with a value of 44.6 g. Least value for average fruit weight was displayed by the genotype H-24 with a value of 20 g.

Values for per plant yield were found to vary from 105 g to 640 g with the genotype Swarna Naveen showing the minimum yield of 105.4 g and the genotype Sakthi with maximum of 641.5 g (Plate 9).

Fruit size was found to range from 2.55cm to 4.6cm with the genotype Swarna Naveen showing the minimum and LE-658 showing the maximum value. All other genotypes were found to differ significantly from the maximum value.

4.2 Molecular Characterization

4.2.1 Isolation and Quantification of Genomic DNA

The protocols suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were used for the isolation of genomic DNA from tender tomato leaves. The quality of DNA isolated by both methods was tested using agarose gel electrophoresis. Better quality DNA indicated by discrete bands and lesser RNA contamination was obtained by the Rogers and Bendich method (Plate 10). The protocol suggested by Doyle and Doyle yielded less discrete DNA bands with higher amount of RNA contamination (Plate 11).

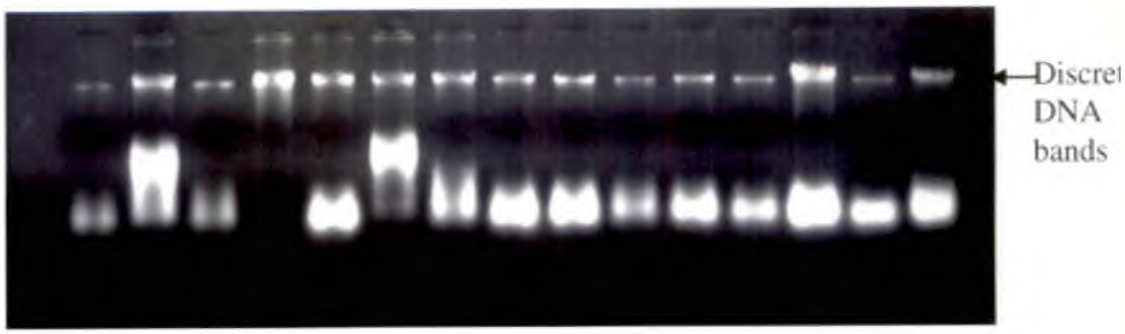


Plate 10. DNA isolated by Rogers and Bendich method

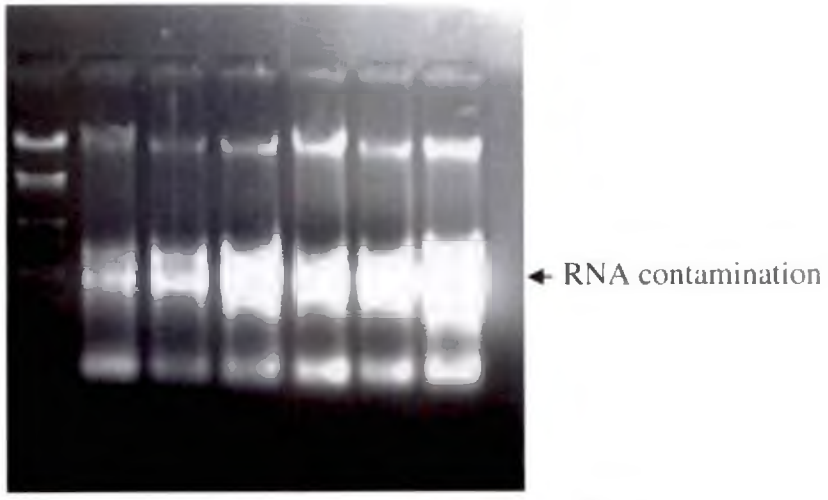


Plate 11. DNA Isolated by Doyle and Doyle method

Plate 12. DNA isolated by Rogers and Bendich method after RNase treatment

RNA contamination in the sample DNA was removed by treatment with RNase A (Sambrook *et al.*, 1989). The electrophoretic profile obtained after RNase treatment revealed clear bands without RNA contamination as shown in Plate 12.

The quality and quantity of DNA isolated from the 15 tomato genotypes by Rogers and Bendich method was estimated by spectrophotometry. Absorbance at 260nm and 280nm were estimated using the Spectronic R Genesys 5 spectrophotometer. The ratio of absorbance at 260nm to that at 280nm ranged from 1.78 to 1.88 indicating relatively pure DNA in the samples. The ratio A_{260}/A_{280} was highest for the genotype LE-474 and least for the genotype LE-638.

Quantity of DNA isolated from each genotype using Rogers and Bendich protocol was calculated. Maximum quantity of DNA was isolated in the genotype LE-640 with a value of 255 µg/g of leaf sample, and minimum quantity of DNA was isolated from the genotype LE-658 with a value of 215.25 µg/g of leaf sample. The quality and quantity of DNA isolated is presented in Table 11.

4.2.2 RAPD Analysis

RAPD assay was carried out with the good quality DNA isolated from all the selected tomato genotypes.

4.2.2.1 Primer Screening

Forty random primers belonging to two different Operon primer series were screened using the DNA isolated from one of the selected tomato genotypes (Hawaii 7998) to select primers showing good amplification for use in genotype screening. The primers screened belonged to the OPS and OPY series.

Table 11 Quantity of DNA isolated from tomato genotypes by Rogers and Bendich method

Genotype	A_{260}	A_{280}	$A_{260/280}$	Quantity (µg/g)
Hawaii 7998	0.328	0.180	1.84	246.00
H-24	0.292	0.163	1.82	219.00
LE-474	0.312	0.169	1.88	234.00
LE-658	0.287	0.157	1.80	215.25
LE-638	0.296	0.164	1.78	222.00
LE-640	0.340	0.187	1.84	255.00
LE-651	0.309	0.171	1.81	231.70
H-86	0.313	0.172	1.82	234.80
Sakthi	0.318	0.174	1.83	238.00
Mukthi	0.313	0.173	1.85	234.75
Anagha	0.301	0.164	1.81	225.75
BL-333-3-1	0.326	0.176	1.83	244.50
BT-218	0.310	0.168	1.84	232.50
Swarna Lalima	0.318	0.175	1.82	238.50
Swarna Naveen	0.320	0.175	1.83	240.00

A. OPS Series

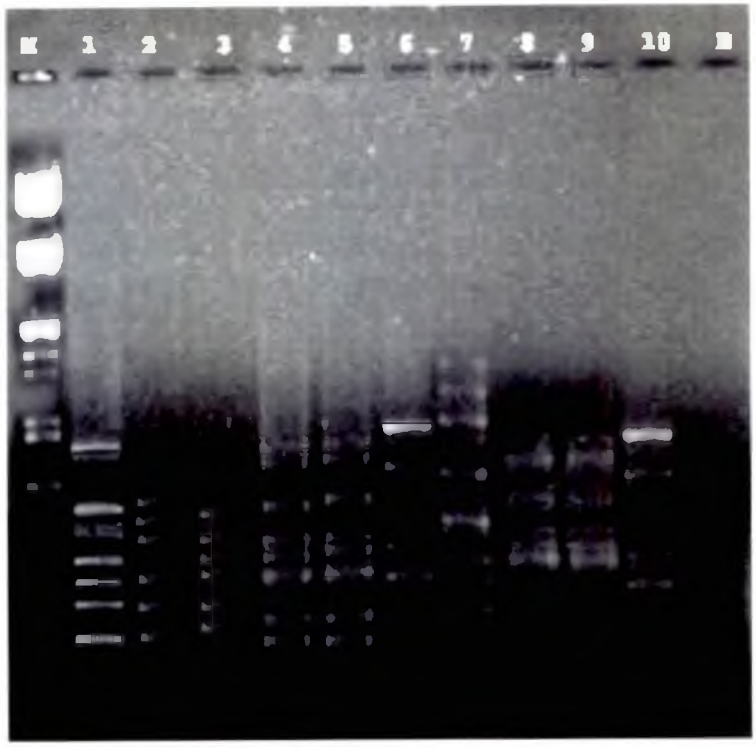
Twenty primers belonging to the OPS series were screened using DNA from the genotype Hawaii 7998. The number of bands obtained using the primers in this series ranged from 5 to 10. The amplification pattern obtained for the primers of this series is shown in Plate 13, and the number of amplification products produced by each primer of the series is given in Table 12. The number of bands produced by the primers ranged from five to ten. All primers with five or more than five amplification products were selected for further RAPD analysis of the tomato genotypes.

B. OPY Series

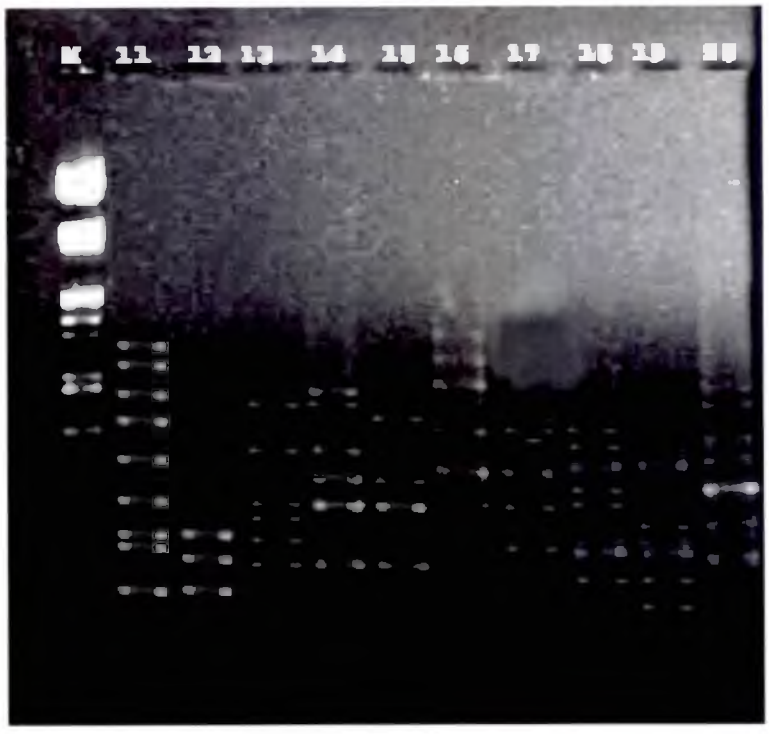
Twenty primers from the OPY Operon primer series were screened prior to selection of primers for genotype screening. The results of screening the 20 primers of the OPY series are presented in Table 13 and Plate 14. The number of bands obtained ranged between two and six. All the primers except OPY 2, OPY 7 and OPY 9 and OPY 11 were used for further RAPD analysis of the 15 tomato genotypes. The primers OPY 2, OPY 7 and OPY 11 gave only two bands during primer screening, so they were rejected for further genotypic screening. The primer OPY 9 did not give any amplicons during primer screening, so this primer was also rejected.

4.2.2.2 RAPD Analysis of Tomato Genotypes

All 15 tomato genotypes differing in reaction to ToLCV disease were analyzed using the 36 random primers belonging to the Operon primer series OPS and OPY. Primers OPS 3, OPS 4, OPS 5, OPS 6, OPS 7, OPS 8, OPS 10, OPS 15 and OPS 20 from the OPS series and OPY 3, OPY 5 and OPY 16 from the OPY series displayed reproducible banding pattern with the number of bands ranging



a. OPS 1 to OPS 10



b. OPS 11 to OPS 20

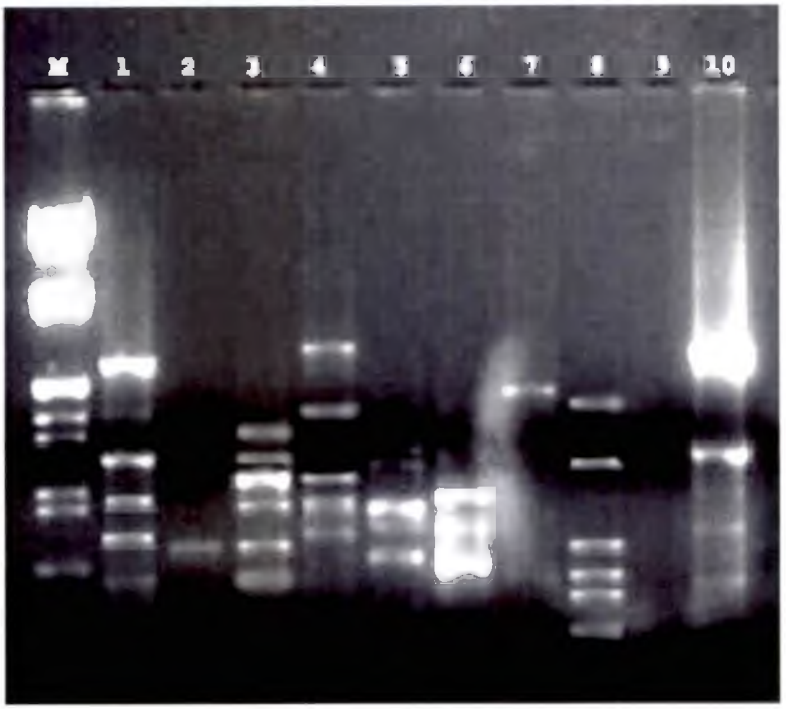
Plate 13. Screening of OPS primers in RAPD assay

Table 12 Number of bands and amplification patterns of OPS primers

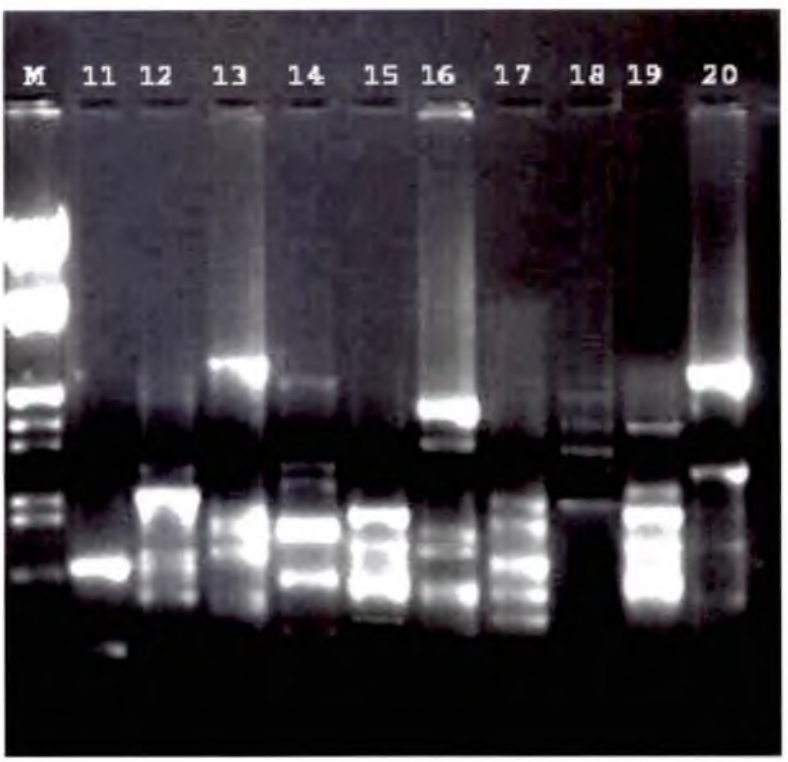
Sl. No	Primer	No. of bands	Amplification pattern
1	OPS 1	7	Good
2	OPS 2	6	Good
3	OPS 3	6	Good
4	OPS 4	8	Good
5	OPS 5	8	Good
6	OPS 6	7	Good
7	OPS 7	10	Good
8	OPS 8	6	Good
9	OPS 9	6	Good
10	OPS 10	6	Good
11	OPS 11	9	Good
12	OPS 12	9	Good
13	OPS 13	7	Good
14	OPS 14	8	Good
15	OPS 15	7	Good
16	OPS 16	10	Good
17	OPS 17	5	Good
18	OPS 18	8	Good
19	OPS 19	8	Good
20	OPS 20	7	Good

Table 13 Number of bands and amplification patterns of OPY primers

Sl. No	Primer	No. of bands	Amplification pattern
1	OPY 1	6	Good
2	OPY 2	2	Average
3	OPY 3	6	Good
4	OPY 4	5	Good
5	OPY 5	5	Good
6	OPY 6	5	Good
7	OPY 7	2	Average
8	OPY 8	6	Good
9	OPY 9	-	Poor
10	OPY 10	5	Good
11	OPY 11	2	Average
12	OPY 12	6	Good
13	OPY 13	5	Good
14	OPY 14	6	Good
15	OPY 15	5	Good
16	OPY 16	5	Good
17	OPY 17	5	Good
18	OPY 18	5	Good
19	OPY 19	6	Good
20	OPY 20	5	Good



a. OPY 1 to OPY 10



b. OPY 11 to OPY 20

Plate 14. Screening of OPY primers in RAPD assay

from five to fourteen and were therefore used for genotype screening. The details of the amplification pattern of the 12 selected primers are as follows:

OPY 3

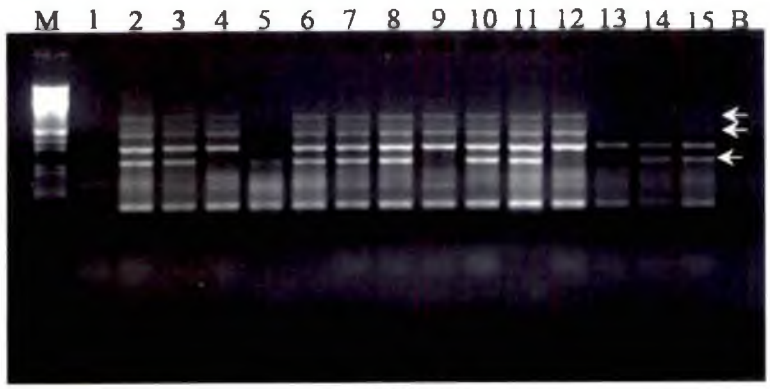
The primer OPY 3 gave nine amplicons after PCR amplification when the products were viewed on agarose gel electrophoresis as shown in Plate 15(a). Seven bands out of the nine were polymorphic. Five amplicons were very distinct and could be easily identified. Among the seven polymorphic bands, the band with molecular weight 1.01 kb was observed in six resistant genotypes and three susceptible genotypes. The primer did not give any amplification in the susceptible genotype BL-333-3-1. The amplicon of molecular weight 0.24 kb was common to all genotypes. The band with molecular weight 1.76 kb was observed in all genotypes except the susceptible genotype Mukthi and resistant genotype LE-651.

OPY 5

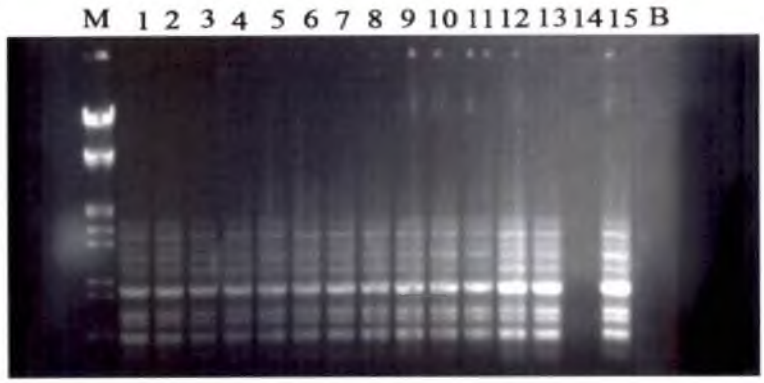
The primer OPY 5 yielded nine amplicons in all genotypes in RAPD assay as shown in Plate 15(b). All the bands were monomorphic in all genotypes except genotype LE-651 in which this primer did not produce any amplification.

OPY 16

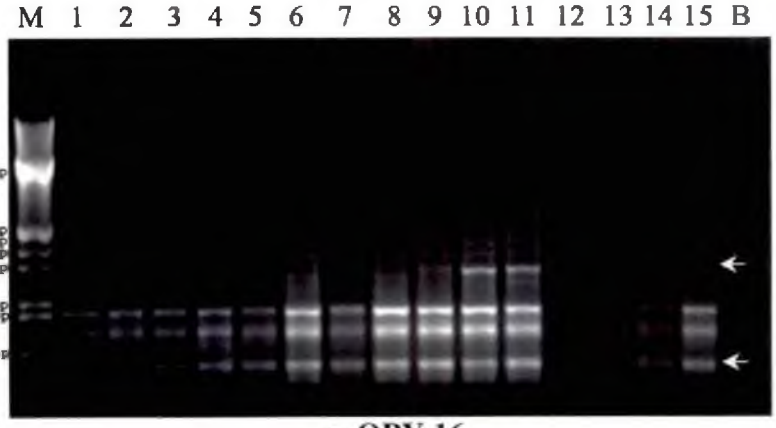
A total of eight amplicons were obtained after DNA amplification with the primer OPY 16 (Plate 15(c)). There were four clear bands among the eight produced. The number of polymorphic bands present was four. Molecular weight of bands ranged from 0.3 to 1.31 kb. Very poor amplification was obtained in case of genotype LE-658 with only one amplicon of molecular weight 0.3 kb.



a. OPY 3



b. OPY 5



c. OPY 16

M-Marker **1**-BL-333-3-1 **2**-H-86 **3**-Hawaii 7998 **4**-Swarna Naveen **5**-Mukthi **6**-Anagha **7**-LE-474 **8**-Swarna Lalima **9**-LE-640 **10**-H-24 **11**-Sakthi **12**-LE-658 **13**-LE 638 **14**-LE-651 **15**-BT-218

*Polymorphism is indicated by arrows

Plate 15. RAPD analysis of tomato genotypes with primers OPY 3, 5 and 16

OPS 3

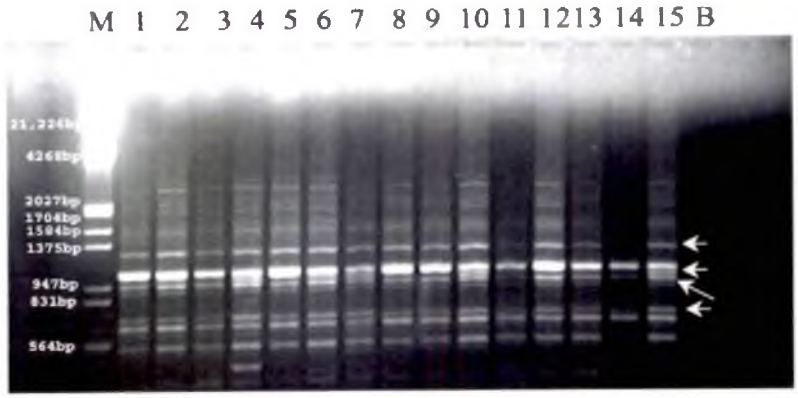
The primer OPS 3 gave maximum number of amplicons (14) among the 12 selected primers. Plate 16(a) shows the profile generated by primer OPS 3. The molecular weight of the amplicons obtained using this primer ranged from 0.2 kb to 2.74 kb. Percentage polymorphism of 79 per cent with a total of 11 polymorphic bands was observed in the amplification profile. The band of size 1.13 kb was present in all genotypes. Eight bands could be clearly identified in the amplification pattern generated. The resistant genotype LE-651 displayed unique banding pattern with only four bands out of the total number of 14. Amplicons common to either resistant or susceptible genotypes were not obtained.

OPS 4

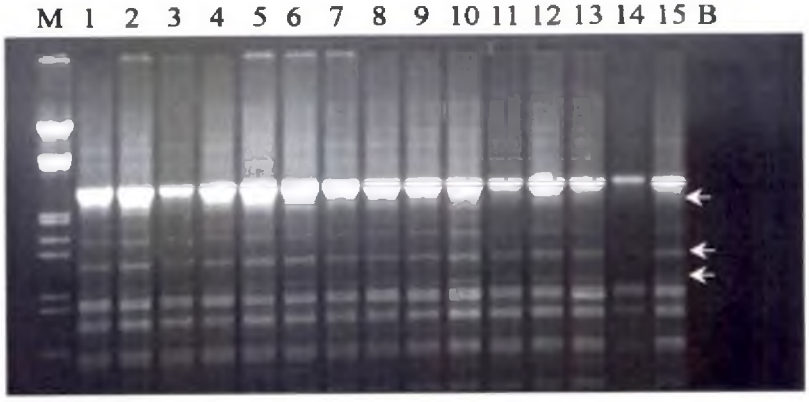
Eight amplicons were observed in the RAPD profile generated by the primer OPS 4 as shown in Plate 16(b), with a total of six polymorphic bands resulting in 75 per cent polymorphism. All eight bands were clearly identifiable. The two monomorphic bands produced were of molecular weight 2.88 kb and 0.91 kb. The resistant genotype LE-651 displayed unique banding pattern with only two bands (monomorphic bands) out of the eight.

OPS 5

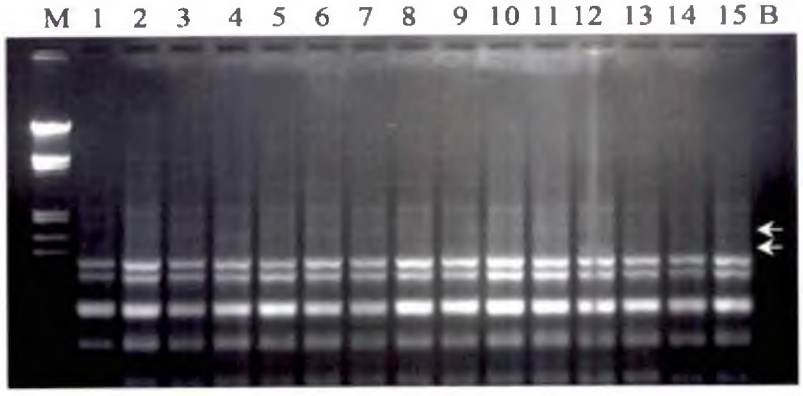
The primer OPS 5 also generated a total of eight amplicons after RAPD analysis of the tomato genotypes as shown in Plate 16(c). A band of molecular weight 2.79 kb was absent in the resistant genotypes Hawaii 7998 and LE-651 and the susceptible genotype BL-333-3-1. Four bands among the eight were very clear and could be easily identified in the RAPD profile generated by this primer. Polymorphism of 38 per cent was obtained by the use of this primer.



a. OPS 3



b. OPS 4



c. OPS 5

M- Marker **1**-BL-333-3-1 **2**-H-86 **3**-Hawaii 7998 **4**-Swarna Naveen **5**- Mukthi **6**-Anagha **7**-LE-474 **8**-Swarna Lalima **9**-LE-640 **10**-H-24 **11**-Sakthi **12**- LE-658 **13**-LE-638 **14**-LE-651 **15**-BT-218

*Polymorphism is indicated by arrows

Plate 16. RAPD analysis of tomato genotypes with primers OPS 3, 4 and 5

OPS 6

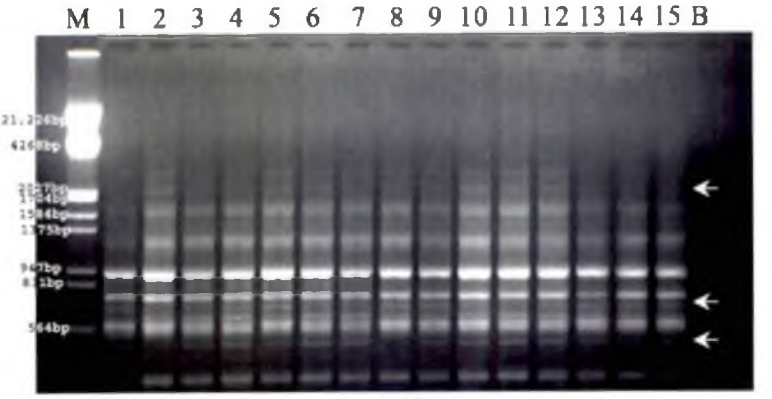
The primer OPS 6 generated 11 bands on RAPD analysis of the tomato genotypes (Plate 17(a)). The bands ranged in molecular weight from 0.18 kb to 1.69 kb. Eight bands were polymorphic giving percentage polymorphism of 73 per cent. Unique banding was observed in the ToLCV susceptible genotype BL-333-3-1. Six bands were clear and easily identifiable. Unique bands shared by resistant/susceptible genotypes were not obtained.

OPS 7

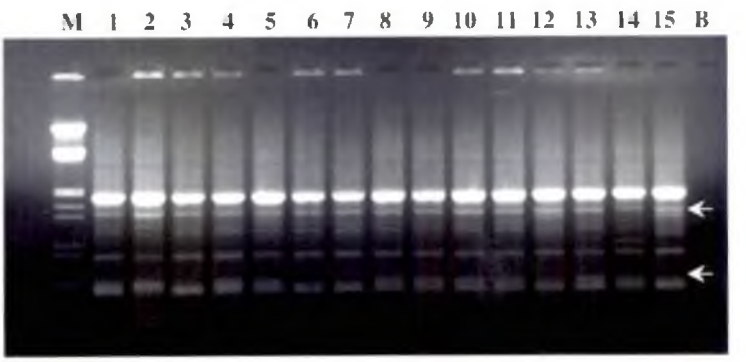
RAPD profile generated by the primer OPS 7 displayed a total of 10 amplicons as observed in Plate 17(b). The amplicons of molecular weight 1.90 kb, 1.50 kb and 0.85 kb were shared by all the tomato genotypes. Amplification profile was not generated for the resistant genotype LE-651. This primer generated polymorphism of 70 per cent. The size of amplicons ranged between 0.3 kb and 1.90 kb. Seven bands were clearly visible. This primer was unable to distinguish between ToLCV resistant and susceptible genotypes.

OPS 8

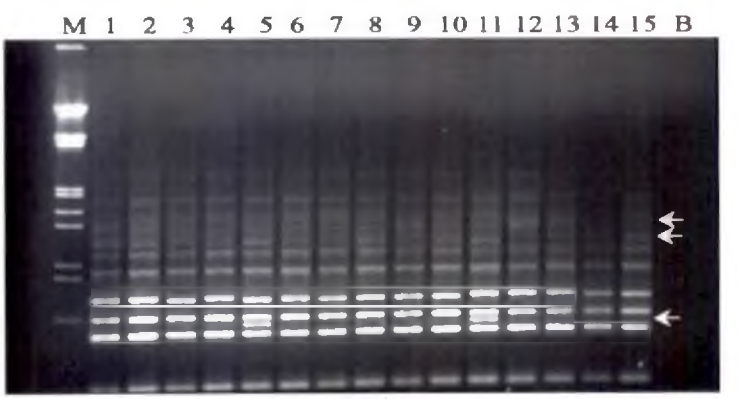
The primer OPS 8 produced a clear RAPD profile for the 15 tomato genotypes with a total of 13 amplicons (Plate 17(c)). Out of 13 bands, eight were found to be polymorphic with 62 per cent polymorphism. One unique band of molecular weight 0.57 kb was shared by two ToLCV susceptible genotypes Sakthi and Mukthi. The band of molecular weight 1.79 kb was absent in all the ToLCV resistant lines LE-640, LE-658, LE-638 and LE-651 obtained from AVRDC. Unique banding pattern was observed in the resistant genotype from AVRDC, LE-651.



a. OPS 6



b. OPS 7



c. OPS 8

M-Marker **1**-BL-333-3-1 **2**-H-86 **3**-Hawaii 7998 **4**-Swarna Naveen **5**-Mukthi **6**-Anagha **7**-LE-474 **8**-Swarna Lalima **9**-LE-640 **10**-H-24 **11**-Sakthi **12**-LE-658 **13**-LE-638 **14**-LE-651 **15**-BT-218

*Polymorphism is indicated by arrows

Plate 17. RAPD analysis of tomato genotypes with primers OPS 6, 7 and 8

OPS 10

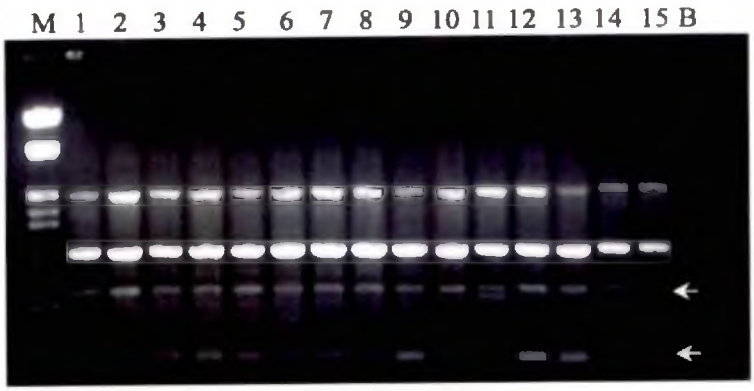
The primer OPS 10 produced five amplicons on RAPD assay of the 15 tomato genotypes (Plate 18(a)). This primer produced minimum number of bands when compared to all other primers selected for the RAPD assay. The bands of size 1.94 kb, 1.01 kb and 0.60 kb were monomorphic and common to all the 15 genotypes. The band of size 0.60 kb was observed in the susceptible genotype Sakthi alone. The percentage polymorphism obtained using the primer OPS 10 was 40 per cent.

OPS 15

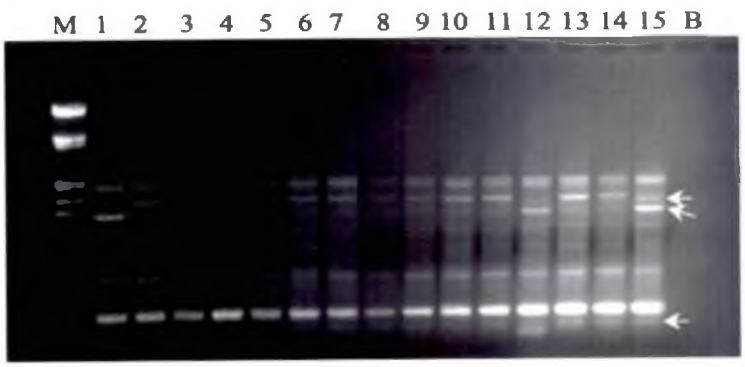
Eleven amplicons were obtained on RAPD assay with the primer OPS 15, out of which seven bands were polymorphic among the genotypes (Plate 18(b)). The amplicon of size 1.61 kb was absent in two susceptible genotypes BT-218 and BL-333-3-1 and in one resistant genotype LE-658. The resistant genotype LE-658 was found to contain one unique band of molecular weight 0.18 kb. The molecular weight of amplicons obtained using this primer ranged from 0.18 kb to 2.08 kb. A total of eight clear bands could be identified in the RAPD profile.

OPS 20

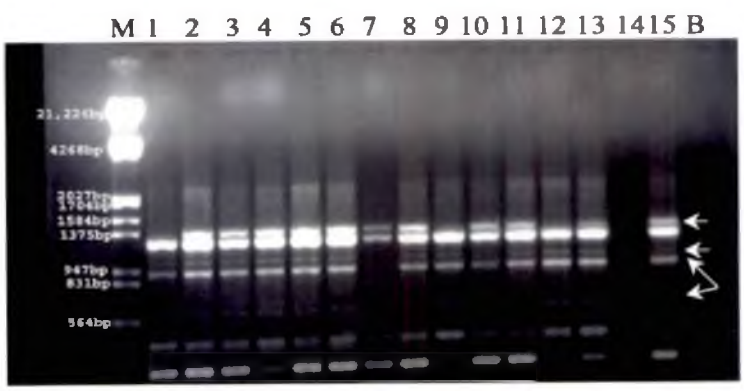
Ten amplicons were observed on the agarose gel for the DNA amplified with the primer OPS 20 (Plate 18(c)). Out of 10, five amplicons were clear and distinct among the varieties. The genotype LE-651 was not amplified with this particular primer. Only a single band of molecular weight 1.24 kb was found shared by all the genotypes. Another band of molecular weight 0.63 kb was shared by all genotypes except LE-474. None of the bands were found shared commonly by the resistant/susceptible genotypes.



a. OPS 10



b. OPS 15



c. OPS 20

M-Marker **1**-BL-333-3-1 **2**-H-86 **3**-Hawaii 7998 **4**-Swarna Naveen **5**-Mukthi **6**-Anagha **7**-LE-474 **8**-Swarna Lalima **9**-LE-640 **10**-H-24 **11**-Sakthi **12**-LE-658 **13**- LE-638 **14**-LE-651 **15**-BT-218

*Polymorphism is represented by arrows

Plate 18. RAPD analysis of tomato genotypes with primers OPS 10, 15 and 20

Though the primers OPY 3, OPY 16, OPS 3, OPS 6, OPS 8, OPS 10, OPS 15 and OPS 20 showed polymorphic banding, the polymorphism was not significant enough to discriminate between genotypes on the basis of resistance to ToLCV disease. The total number of amplification products produced by each primer and the number of polymorphic bands obtained are summarized in Table 14.

Resolving power of the 12 random decamer primers selected for the study was calculated using the formula $Rp = \Sigma Ib$, where 'Ib' is the 'band informativeness' calculated as $Ib = 1 - (2 \times |0.5-p|)$, where 'p' is the proportion of genotypes containing the band. The resolving power of the 12 primers ranged from 0.80 for the primer OPS 10 to a value of 6.16 for the primer OPS 3. The resolving power calculated for each of the 12 selected primers in given in Table 14.

4.2.2.3 Analysis of Banding Patterns

The amplification profiles obtained on RAPD analysis of the tomato genotypes were compared and scored as zero and one based on the absence or presence of bands. The data was analyzed using the Numerical Taxonomy System of Multivariate Statistical Program (NTSyS) software package. The pair-wise similarity matrix was constructed using the SIMQUAL program (Table 15). The results indicated that the similarity coefficients between the genotypes ranged from 0.46 to 0.92 with maximum similarity among the ToLCV resistant genotypes Anagha and H-24, and the minimum similarity existed between the resistant genotypes LE-658 and LE-651.

The genotype LE-651 was found to be a unique genotype with low level of similarity to all the other tomato genotypes included in the study. The similarity values between the genotype LE-651 and all other genotypes ranged from 0.46 with the resistant genotype LE-658 to 0.64 with the resistant genotype LE-638.

Table 14 Amplification products, polymorphic bands and resolving power of selected RAPD primers

Sl. No.	Primer	No:of bands	No:of Polymorphic bands	Per cent polymorphism	Resolving Power
1	OPY 3	9	7	78	4.14
2	OPY5	9	0	0	1.26
3	OPY 16	8	4	50	4.00
4	OPS 3	14	11	79	6.16
5	OPS 4	8	6	75	2.02
6	OPS 5	8	3	38	1.34
7	OPS 6	11	8	73	3.78
8	OPS 7	10	7	70	3.40
9	OPS 8	13	8	62	3.40
10	OPS 10	5	2	40	0.80
11	OPS 15	11	7	64	3.78
12	OPS 20	10	8	80	3.60

Table 15 Similarity values based on RAPD profiling of tomato genotypes

Rows\Cols	BL333-3-1	H-86	Hawaii_79	Swarna_Na	Mukthi	Anagha	LE_474	Swarna_La	LE_640	H-24	Sakthi	LE_658	LE_638	LE_651	BT-218
BL333-3-1	1.0000000														
H-86	0.7526882	1.0000000													
Hawaii_79	0.7356322	0.8118812	1.0000000												
Swarna_Na	0.7391304	0.8640777	0.8453608	1.0000000											
Mukthi	0.7446809	0.8834951	0.8282828	0.8811881	1.0000000										
Anagha	0.7473684	0.8598131	0.8058252	0.8224299	0.8240741	1.0000000									
LE_474	0.7032967	0.8252427	0.8617021	0.8217822	0.8058252	0.8365385	1.0000000								
Swarna_La	0.7888889	0.8834951	0.8469388	0.8446602	0.8640777	0.8761905	0.8235294	1.0000000							
LE_640	0.7727273	0.7980769	0.7755102	0.7941176	0.7619048	0.8095238	0.7722772	0.8316832	1.0000000						
H-24	0.7142857	0.8785047	0.7735849	0.8411215	0.8425926	0.9245283	0.8980952	0.8598131	0.7943925	1.0000000					
Sakthi	0.6989247	0.7685185	0.7281553	0.7314815	0.7339450	0.8130841	0.7428571	0.7830189	0.7500000	0.8317757	1.0000000				
LE_658	0.6770833	0.8037383	0.7307692	0.8000000	0.7850467	0.7981651	0.7619048	0.7522936	0.7864078	0.8165138	0.7090909	1.0000000			
LE_638	0.7701149	0.7403846	0.7319588	0.7524752	0.7378641	0.7358491	0.7300000	0.7378641	0.7916667	0.7383178	0.7425743	0.7623762	1.0000000		
LE_651	0.5625000	0.5180723	0.5405405	0.5125000	0.5000000	0.4772727	0.5250000	0.5121951	0.5657895	0.5000000	0.5512821	0.4642857	0.6417910	1.0000000	
BT-218	0.7473684	0.8411215	0.7523810	0.8037383	0.8055556	0.8691589	0.7830189	0.8396226	0.7904762	0.8678505	0.7454545	0.7962963	0.7500000	0.5119048	1.0000000

1. BL-333-3-1, **2.** H-86, **3.** Hawaii 7998, **4.** Swarna Naveen, **5.** Mukthi, **6.** Anagha, **7.** LE-474, **8.** Swarna Lalima, **9.** LE-640, **10.** H-24, **11.** Sakthi, **12.** LE-658, **13.** LE-638, **14.** LE-651, **15.** BT-218.

The dendrogram prepared using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) by the help of the NTSyS software package (Fig. 1) revealed maximum similarity of 92 per cent between the resistant genotypes Anagha and H-24. The ToLCV resistant genotypes Hawaii 7998 and LE-474 were clustered together with similarity of around 87 per cent.

The resistant genotypes from AVRDC, Taiwan namely LE-640 and LE-658 were grouped together in a single sub-cluster showing similarity of approximately 79 per cent. ToLCV susceptible genotypes Mukthi, Swarna Naveen and Swarna Lalima were grouped with ToLCV resistant genotype H-86 by a similarity of approximately 87 per cent with the genotypes Mukthi and H-86 being most similar.

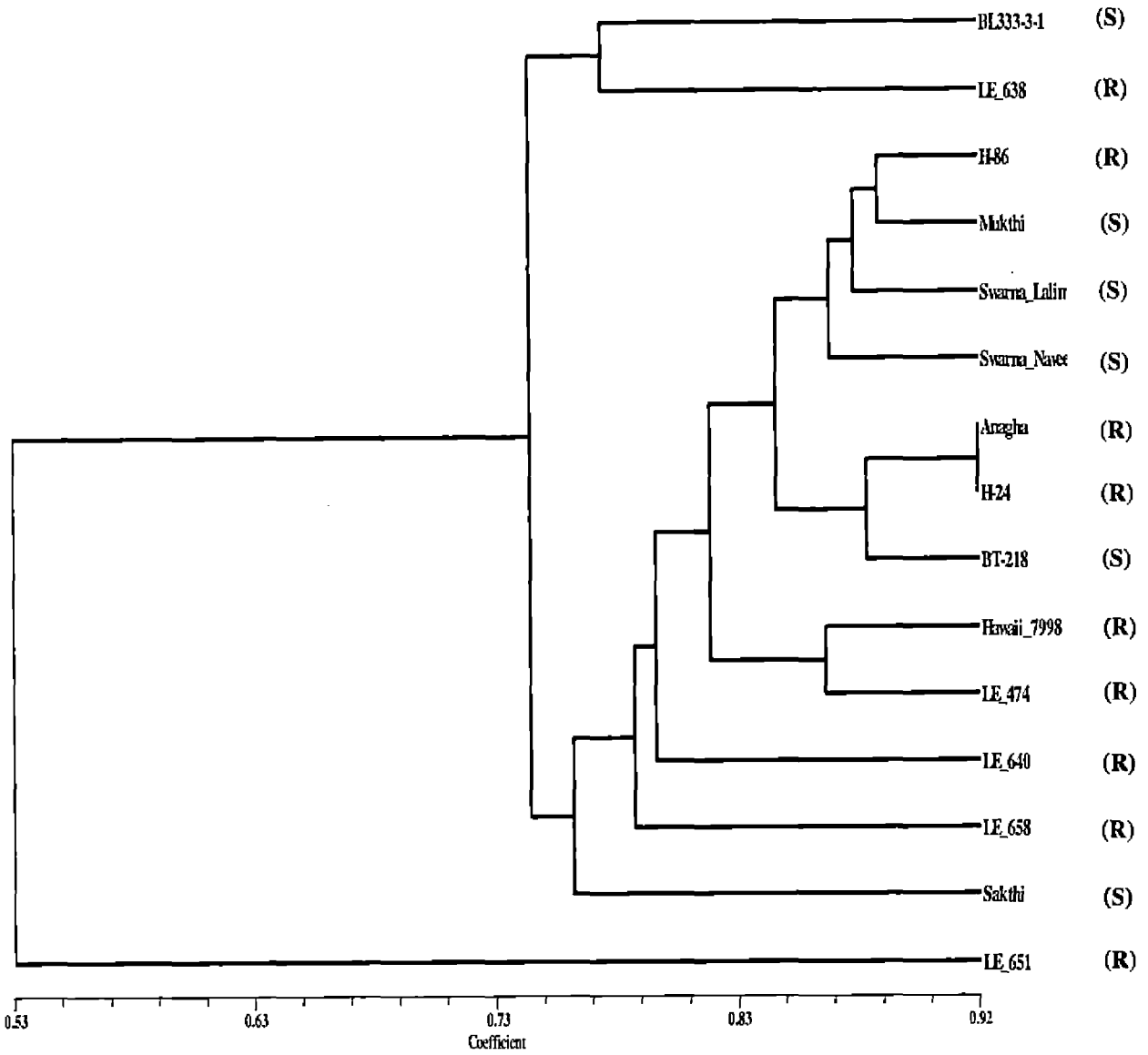
The resistant genotype LE-651 was separated from all other genotypes by a value of about 47 per cent. The dendrogram revealed high degree of relatedness among the 15 tomato genotypes selected for the study.

4.2.3 AFLP Analysis

AFLP analysis was carried out with the DNA samples isolated from 10 selected tomato genotypes, which included five resistant (Hawaii 7998, H-24, H-86, LE-474, Anagha) and five susceptible genotypes (Swarna Naveen, Swarna Lalima, Sakthi, Mukthi, BL-333-3-1).

The DNA samples were pre-amplified and the diluted pre-amplified DNA were used for selective amplification with radiolabled *Eco*RI and *Mse*I primers.

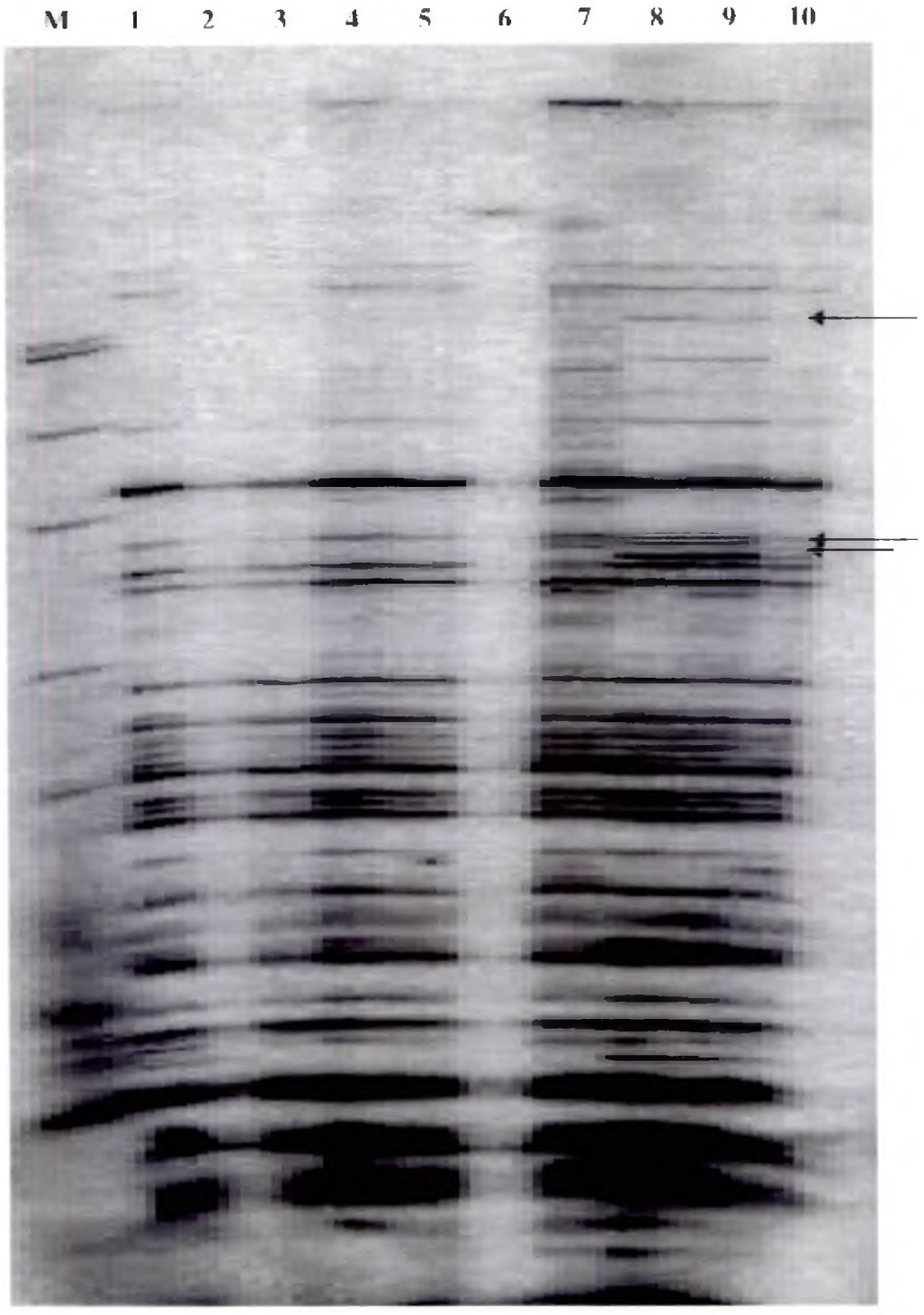
Five different combinations of *Eco*RI and *Mse*I primers were used for AFLP analysis. The banding patterns were visualized and documented using a Phosphor Imager FLA-5100 system (Plate 19, 20, 21, 22 and 23).



(R) – Resistant to ToLCV

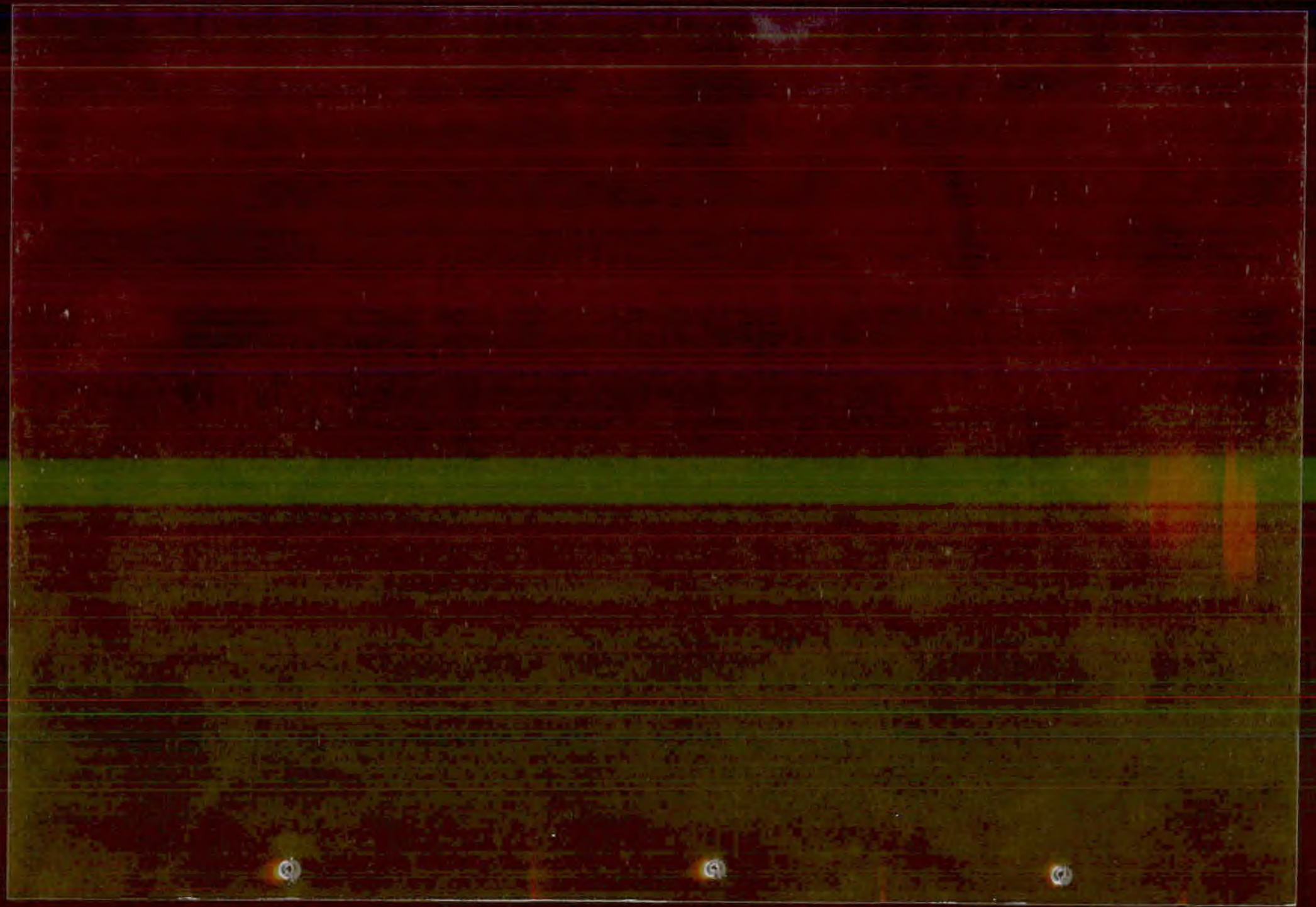
(S) – Susceptible to ToLCV

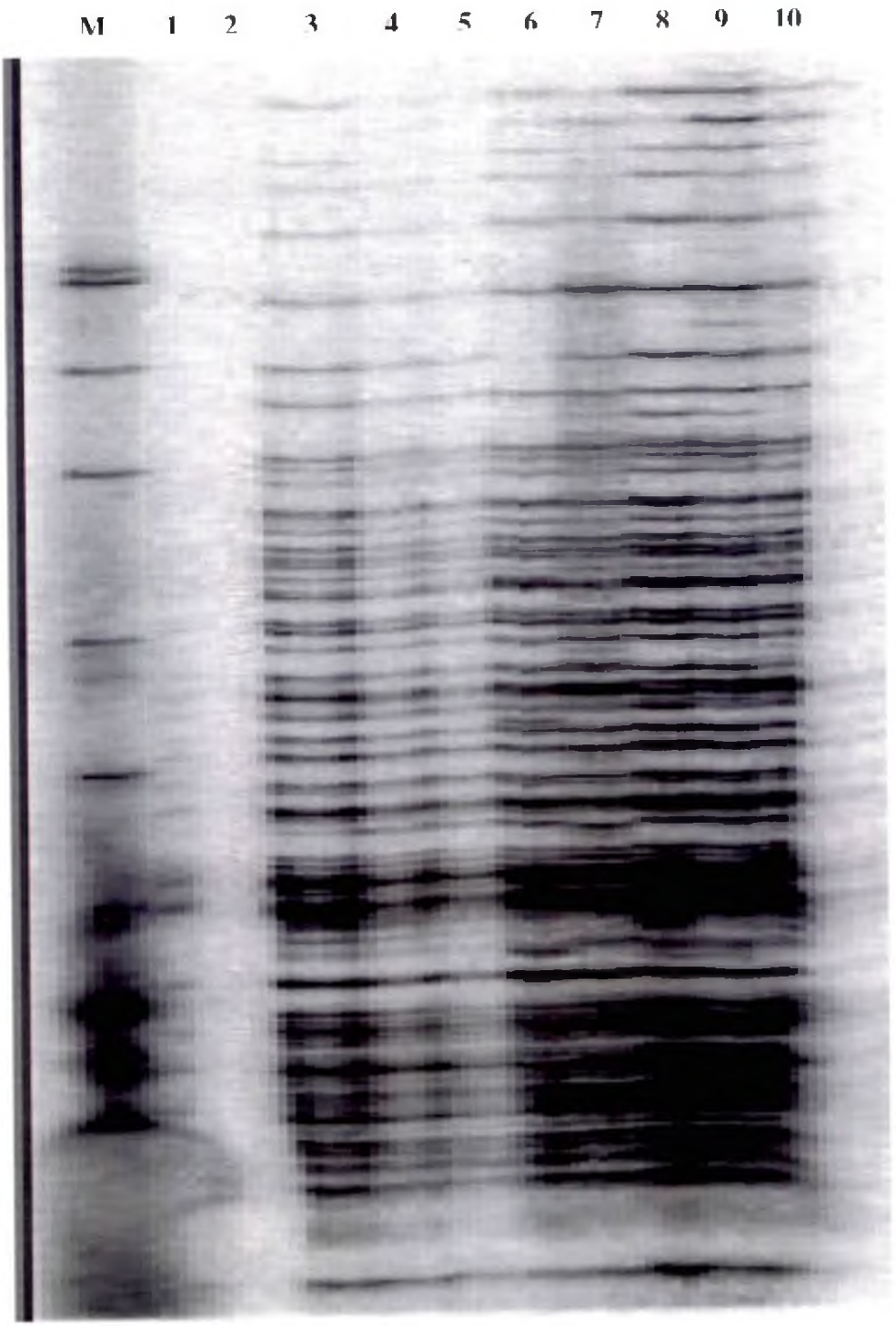
Fig. 1 Dendrogram derived from RAPD analysis of 15 tomato genotypes



M. Marker **1.** Hawaii 7998 **2.** H-24 **3.** H-86 **4.** LE-474 **5.** Anagha **6.** Swarna Naveen **7.** Swarna Lalima **8.** Sakthi **9.** Mukthi **10.** BL-333-3-1

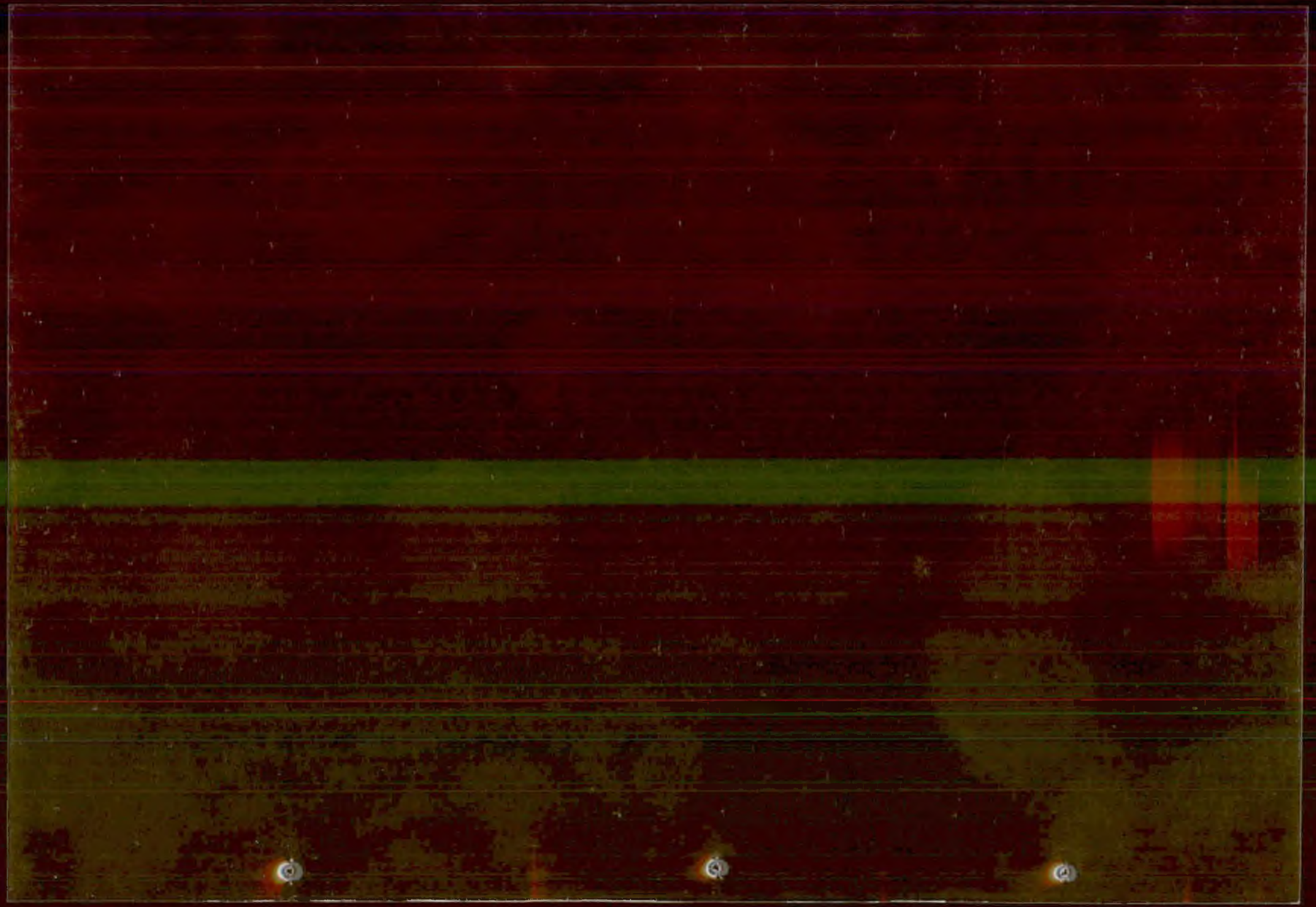
Plate 19. AFLP profile of selected tomato genotypes with EAAG/MCAC primer pair

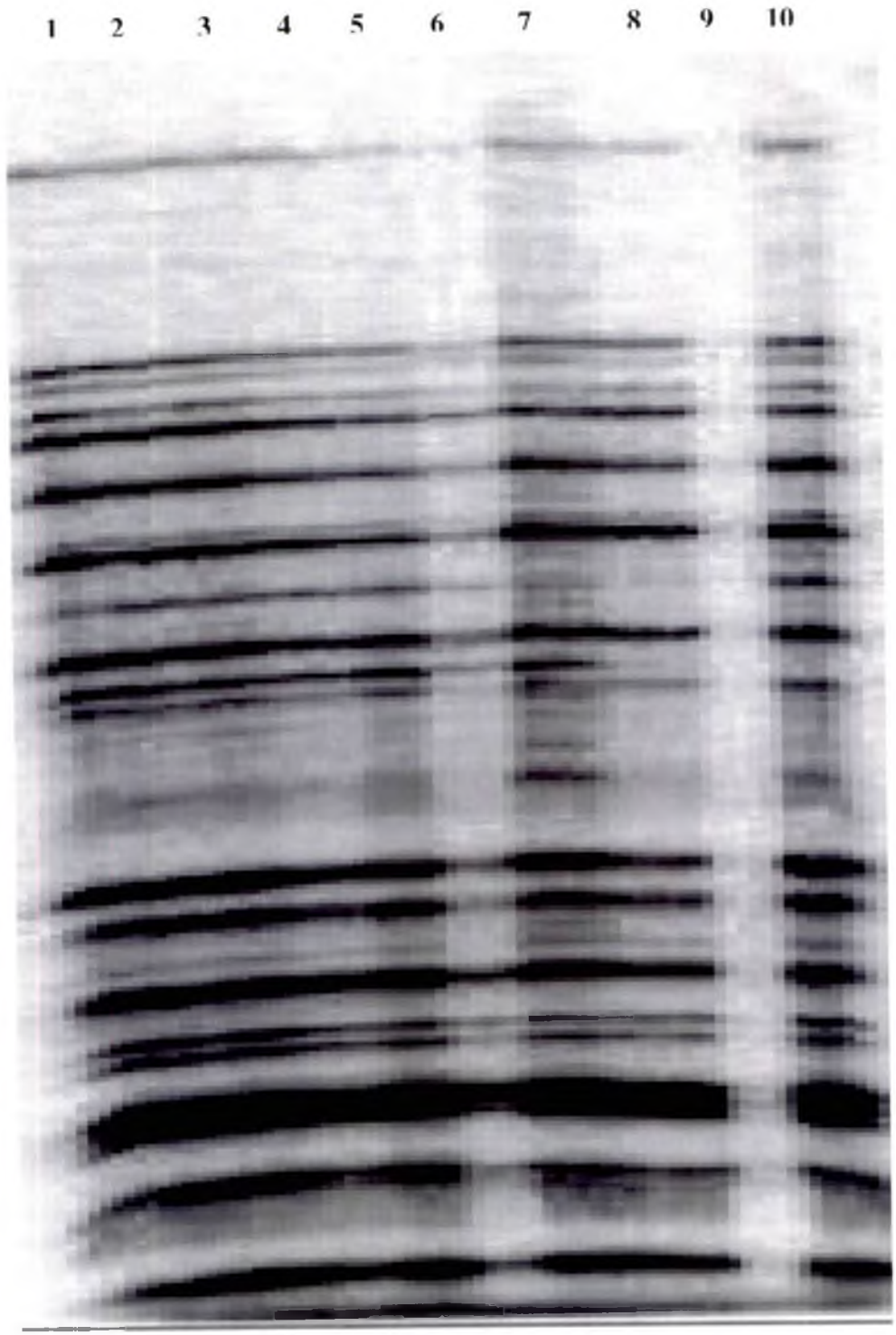




M. Marker **1.** Hawaii 7998 **2.** H-24 **3.** H-86 **4.** LE-474 **5.** Anagha **6.** Swarna Naveen **7.** Swarna Lalima **8.** Sakthi **9.** Mukthi **10.** BL-333-3-1

Plate 20. AFLP profile of selected tomato genotypes with EACC/MCTC primer pair





1. Hawaii 7998 **2.** H-24 **3.** H-86 **4.** LE 474 **5.** Anagha
6. Swarna Naveen **7.** Swarna Lalima **8.** Sakthi **9.** Mukthi **10.** BL333-3-1

Plate 21. AFLP profile of selected tomato genotypes with EAGC/MCTA primer pair

1. Hawaii 7998 **2.** H-24 **3.** H-86 **4.** LE 474 **5.** Anagha
6. Swarna Naveen **7.** Swarna Lalima **8.** Sakthi **9.** Mukthi **10.** BL-333-3-1

Plate 22. AFLP profile of selected tomato genotypes with EACG/MCAC primer pair

1. Hawaii 7998 **2.** H-24 **3.** H-86 **4.** LE 474 **5.** Anagha
6. Swarna Naveen **7.** Swarna Lalima **8.** Sakthi **9.** Mukthi **10.** BL-333-3-1

Plate 23. AFLP profile of selected tomato genotypes with EAGG/MCAA primer pair

4.2.3.1 Analysis of Banding Patterns in AFLP Assay

The banding patterns were scored as zero and one based on the absence and presence of bands. The five primer combinations used and the total number of bands, number of polymorphic bands and the percentage polymorphism obtained for each primer pair are shown in Table 16. A total of 241 fragments were detected after AFLP assay of the 10 selected genotypes using five primer combinations. Out of 241 fragments detected, the total number of polymorphic bands detected was 122.

Maximum number of amplicons was detected using the primer combination EACC/MCTC (71) and minimum number of amplicons was detected using the combination EAGC/MCTA (28). The percentage polymorphism obtained for the primers ranged from 29.5 per cent to 87.2 per cent. The primer combination EAAG/MCAC gave maximum number of polymorphic bands (41) and highest per cent polymorphism, while the primer combination EAGG/MCAA showed minimum number of polymorphic bands (13) and percentage polymorphism (29.5).

Three amplicons were observed in the ToLCV susceptible genotypes Sakthi, Mukthi and Swarna Lalima using the primer combination EAAG/MCAC (Plate 19).

The scored data was analyzed using the Numerical Taxonomy System of Multivariate Statistical Program (NTSyS) software package and pair-wise similarity matrix was constructed using the SIMQUAL program (Table 17). Clustering was done and a dendrogram was prepared using UPGMA (Fig. 2).

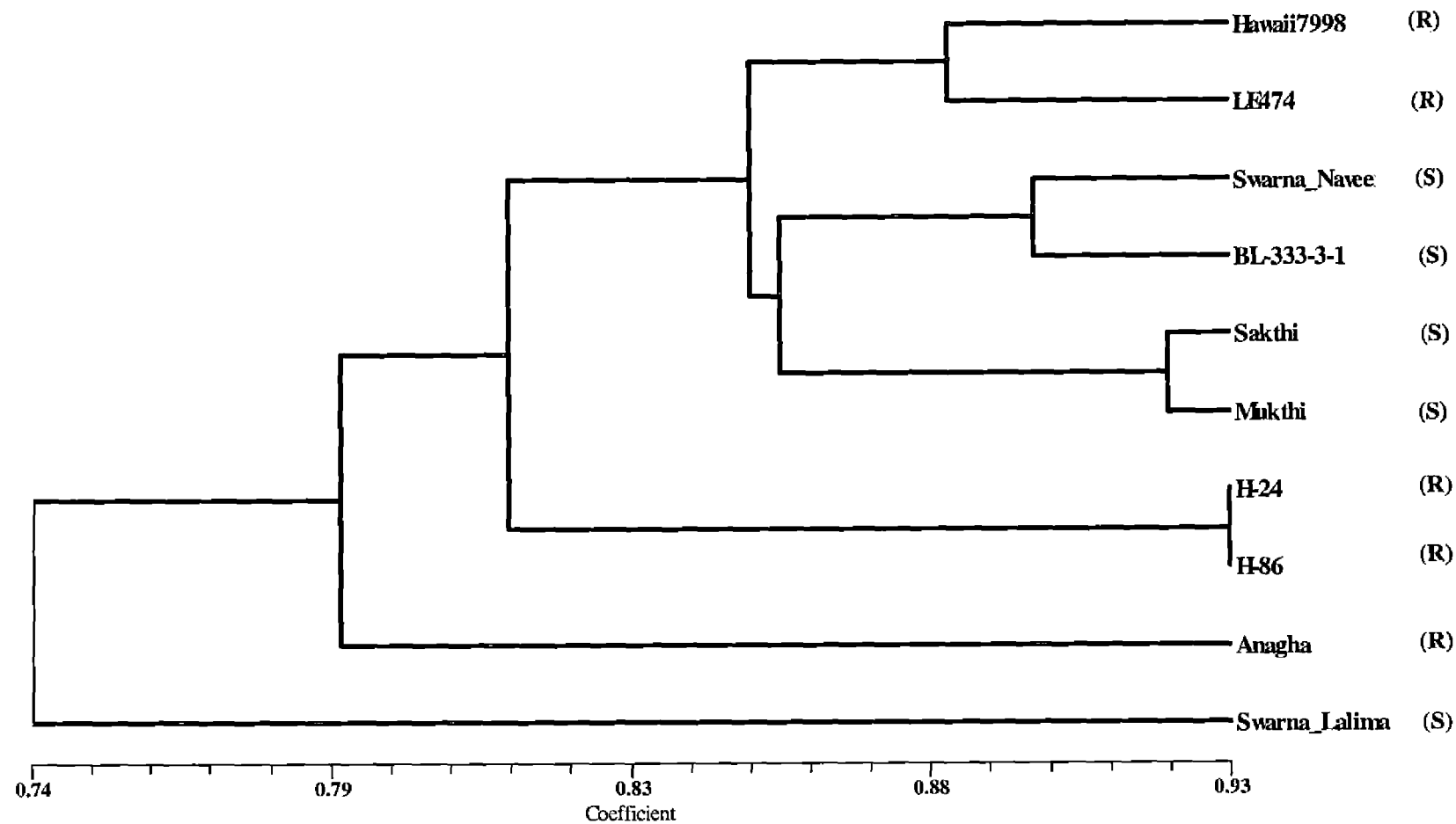
The coefficient of similarity obtained from the pair-wise similarity matrix ranged from 0.66 to 0.92 (Table 17) indicating minimum similarity between the susceptible genotype Swarna Lalima and the resistant genotype H-24, and

Table 16 Primer combinations and amplification patterns of AFLP primers

Sl. No:	Primer Combination	No: of bands	No: of polymorphic bands	Per cent polymorphism
1	EAGG+MCAA	44	13	29.5
2	EACG+MCAC	51	23	45.1
3	EACC+MCTC	71	33	46.5
4	EAAG+MCAC	47	41	87.2
5	EAGC+MCTA	28	12	42.9

Table 17 Similarity values based on AFLP profiling of tomato genotypes

Rows\Cols	Hawai1799	H-24	H-86	LE474	Anagha	Swarna_Na	Swarna_La	Sakthi	Mukthi	BL-333-3-
Hawai1799	1.0000000									
H-24	0.8394161	1.0000000								
H-86	0.8613139	0.9280000	1.0000000							
LE474	0.8843537	0.7891156	0.8146341	1.0000000						
Anagha	0.8500000	0.7753623	0.7512690	0.8050000	1.0000000					
Swarna_Na	0.8684211	0.8771930	0.8587571	0.8465909	0.7894737	1.0000000				
Swarna_La	0.7453416	0.6687500	0.7333333	0.7652174	0.6769912	0.7891892	1.0000000			
Sakthi	0.8943662	0.7957746	0.8164251	0.8578199	0.7892157	0.8651685	0.7596567	1.0000000		
Mukthi	0.8416667	0.7024793	0.7460317	0.8125000	0.7569061	0.8645161	0.7439614	0.9184783	1.0000000	
BL-333-3-	0.8551724	0.8345324	0.8557214	0.8523810	0.8009950	0.8977273	0.7929515	0.8625592	0.8404255	1.0000000



(R) – Resistant to ToLCV

(S) – Susceptible to ToLCV

Fig. 2 Dendrogram derived from AFLP analysis of 10 tomato genotypes using five primer combinations

maximum similarity between the ToLCV resistant genotypes H-24 and H-86, both obtained from the Indian Institute of Vegetable Research, Varanasi.

The susceptible genotype Swarna Lalima displayed the lowest similarity values (0.66 to 0.78) with the rest of the genotypes involved in the study. Low similarity was also observed between the susceptible genotype Mukthi and resistant genotype H-24 (0.70), Mukthi and the resistant genotype H-86 (0.74) and Mukthi and the susceptible genotype Swarna Lalima (0.74).

The dendrogram grouped the ToLCV resistant genotypes H-24 and H-86 together with highest similarity of 93 per cent (Fig. 2). Other ToLCV resistant genotypes Hawaii 7998 and LE-474 were grouped together with similarity of approximately 89 per cent. Susceptible genotypes Sakthi and Mukthi and Swarna Naveen and BL-333-3-1 formed two separate groups with similarity of 92 per cent and 90 per cent respectively.

All genotypes except resistant genotype Anagha and susceptible genotype Swarna Lalima were grouped under a single cluster. The genotype Anagha showed around 79 per cent similarity with this large cluster of genotypes. The susceptible genotype Swarna Lalima was different from all other genotypes taken as a whole by a value of about 26 per cent.

Discussion

5. DISCUSSION

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops grown in India. The English traders of East India Company introduced it to India in 1822. The crop occupies an area of 4.1 million hectares with a productivity of 26 MT/ha in the world. In India, tomatoes are cultivated in an area of 0.50 million hectares with a productivity of 17.4 MT/ha (Chamber *et al.*, 2006). The major tomato producing states in the country are Uttar Pradesh, Karnataka, Maharashtra, Punjab and Haryana.

One of the reasons for low productivity of tomato in India is the occurrence of leaf curl, a serious disease caused by the Tomato Leaf Curl Virus (ToLCV), a virus belonging to the geminivirus group, which causes yield losses between 70 and 100 per cent. Whitefly transmitted geminiviruses belonging to the genus Begomovirus of the family *Geminiviridae* have emerged as devastating pathogens of crops worldwide. Each year ToLCV causes extensive damage to tomato crops all over the world. Adoption of disease resistant varieties or hybrids is the most feasible way to combat this problem. Several ToLCV resistant tomato varieties and F_1 hybrids are being commercially cultivated in India. However, the existence of a number of strains of the virus has restricted the availability of a single variety resistant to all the prevalent ToLCV strains.

In this context, the work entitled "Molecular characterization of tomato (*Solanum lycopersicon* L.) with special reference to tomato leaf curl virus (ToLCV) resistance" was undertaken with the following targets:

1. Screening genotypes reported resistant/susceptible to tomato leaf curl virus from different regions for their response to ToLCV under conditions prevalent in Kerala to identify the resistant/susceptible ones.
2. Molecular characterization of the selected genotypes with respect to ToLCV resistance using RAPD and AFLP marker systems.

The results obtained from the study are dealt with and discussed in this chapter.

5.1 Reaction of Genotypes to ToLCV

Leaf curl caused by ToLCV is a serious production constraint of tomato especially in warm-dry environments in the Indian sub-continent and many other Asian countries.

Fifteen genotypes selected for the study were screened for their reaction to ToLCV under natural conditions in pots and field during December to February, which is the peak season for ToLCV incidence in Kerala. The genotypes included in the study were LE-658, LE-638, LE640 and LE-651 reported to be resistant from AVRDC, Taiwan; H-24 and H-86, resistant genotypes reported from IIVR, Varanasi; Hawaii 7998 obtained from HPKV, Palampur reported to be resistant after field screening conducted at the Department of Olericulture, Kerala Agricultural University (KAU); Anagha and LE-474 reported to be resistant from KAU, Vellanikkara; susceptible genotypes BL-333-3-1 from HPKV, Palampur; Swarna Lalima and Swarna Naveen from HARP, Ranchi; and Sakthi and Mukthi, susceptible genotypes reported from the Kerala Agricultural University, Vellanikkara. The soil in both pots and field were sterilized by the application of formaldehyde (1:30) to prevent the occurrence of bacterial wilt disease that would interfere with the ToLCV screening process. The acidic soil and humid conditions in Kerala greatly favours bacterial wilt incidence due to infection by *Ralstonia solanacearum*, and the inoculum present in the soil is the main source of infection. Soil sterilization with formalin is reported to control bacterial wilt by reducing the soil inoculum.

Genotypes were scored according to the 0 to 4 scale suggested by Banerjee and Kalloo (1998) based on the symptoms of leaf curl produced. Singh *et al.* (2003) also reported the categorization of tomato genotypes based on their

reaction to ToLCV according to the same score chart suggested by Banerjee and Kalloo.

After scoring, per cent disease incidence, per cent disease severity and coefficient of infection were calculated for each genotype in both pot culture and field experiment. On the basis of coefficient of infection, genotypes were grouped into different categories with respect to disease reaction. The genotypes Hawaii 7998, H-24, H-86, Anagha, LE-658 and LE-651 were completely free from disease in both pot and field experiments. Genotype LE-474 showed mild infection (4.6) in field condition but was completely free of disease in pot culture experiment. The coefficient of infection ranged between 42.5 and 82.5 in pot culture and 34 and 83.8 in field in the susceptible genotypes BL-333-3-1, BT-218, Swarna Lalima, Swarna Naveen, Sakthi and Mukthi.

Resistance of the genotype H-24 to ToLCV has been reported by other researchers also. Kalloo and Banerjee (2000) studied the performance of the genotype 'H-24' under field condition and by artificial inoculation and reported that H-24 can be grown in leaf curl infested area and can also be used as a tolerant breeding line. Thamburaj and Singh (2001) reported the existence of the leaf curl resistant genotype H-24 from Varanasi. Veeraragavathatham *et al.* (2002) reported the use of the genotype H-24 as a good parent for breeding programs to develop ToLCV resistant lines. The resistance of the cultivar H-24 to ToLCV is reported to be due to the possession of a resistant gene from the wild species *Solanum lycopersicum f. glabratum* '86093' (Kumar *et al.*, 2002). The importance of the genotype H-86 as a source of resistance to ToLCV and its application as a resistant parent in breeding programs was discussed by Gururaj *et al.* (2002). The present study confirms the resistance of H-24 and H-86 in Kerala conditions too. The genotypes LE-658, LE-638, LE-651 and LE-640 obtained from AVRDC as resistant lines ensured their resistance to ToLCV under Kerala conditions also.

The resistance of genotypes in the study to ToLCV could be due to the incorporation of resistance genes from wild species like *Lycopersicon hirsutum, Lycopersicon chilense, Lycopersicon peruvianum* or *Lycopersicon pimpinellifolium.* There are reports of resistance to leaf curl in accessions belonging to these species by different researchers. Narasegowda *et al.* (2003) reported accessions of the wild species *Lycopersicon hirsutum,* LA 1777 and PI 390659 to be good sources of resistance to ToLCV (India) and TYLCV (Tomato Yellow Leaf Curl Virus). In a study involving screening for resistance to TYLCV, an accession of *Lycopersicon chilense*, 'LA 1969' was reported to show high level of resistance to the disease (Zakay *et al.*, 1991). Hanson *et al.* (2000) reported the resistance of *Lycopersicon hirsutum f. glabratum* 'B6013' to ToLCV. Sadashiva *et al.* (2006) identified eight tomato accessions, one belonging to *Lycopersicon hirsutum,* two to *Lycopersicon peruvianum* and the remaining from *Solanum lycopersicum* to be completely free from ToLCV incidence under field screening and artificial inoculation using viruliferous white flies. Resistance of accessions of *Lycopersicon cheesmanii, Lycopersicon peruvianum* and *Lycopersicon pimpinellifolium* to ToLCV was reported by Tripathi and Varma (2003).

The resistance of certain genotypes in the study may be due to the inability of whiteflies to feed on the host or due to interference of the plant with the life cycle of the virus as in case of resistance to TYLCV reported by Zakay *et al.* (1991). The mechanism of resistance in the genotypes selected for the study could be the blockage of long distance viral movement in the vascular tissues along with development of hypersensitive response (HR) that developed as a secondary defense as reported by Seo *et al.* (2004) in common bean (*Phaseolus vulgaris* L.) in relation to resistance to the geminivirus Bean Dwarf Mosaic Virus (BDMV).

In the present study all the genotypes reported to be resistant from different locations in India were found to be resistant to ToLCV in Kerala also. The source of resistance in the AVRDC lines cannot be interpreted since it is not disclosed by the sender and is at present named only as accession numbers.

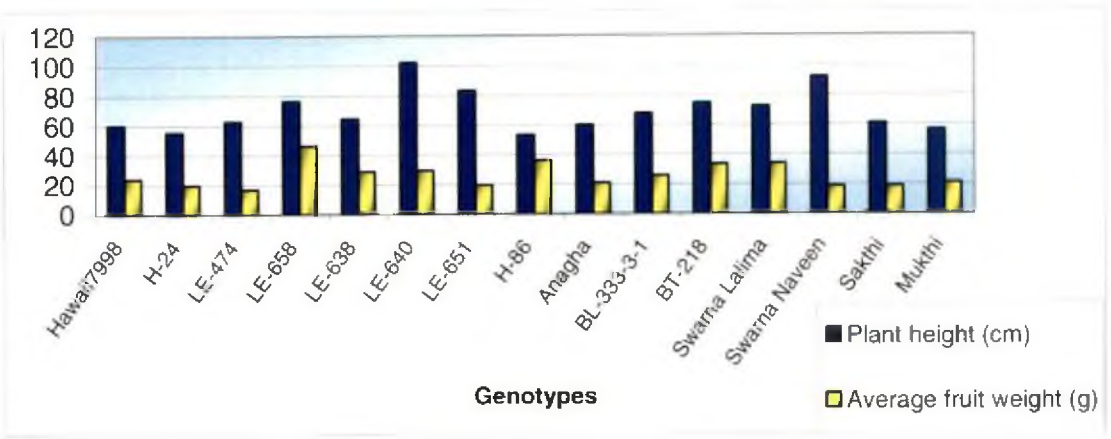
Resistance observed in the field and pot experiments was confirmed through artificial inoculation by cleft grafting, which also showed the resistance of Hawaii 7998, H-24, H-86, Anagha, LE-638, LE-658, LE-651 and LE-640 to ToLCV infection. Since ToLCV is sap transmissible, graft inoculation is one of the commonly adopted methods of artificial inoculation in addition to whitefly-transmission and agroinoculation. Confirmation of resistance of genotypes to ToLCV, Tomato Spotted Wilt Virus (TSWV) and Tomato Yellow Leaf Curl Virus (TYLCV) in tomato by grafting method has been reported by Kumar *et al.* (2002), Tavella *et al.* (2005) and Delatte *et al.* (2006).

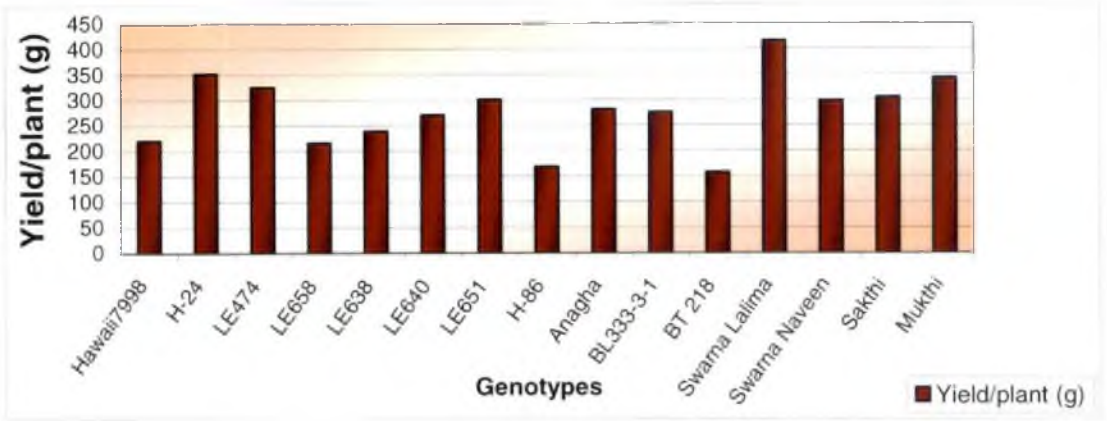
5.2 Comparison of Genotypes based on Biometric Characters

Fifteen genotypes selected on the basis of their reported resistance/susceptibility to ToLCV under conditions prevalent elsewhere were compared on the basis of growth parameters like plant height, internode length, number of primary branches, average fruit weight, fruit size and per plant yield in field and pot culture conditions.

Significant differences were observed among the different genotypes in the case of all of the biometric characters like plant height, internode length, plant spread/number of primary branches, average fruit weight and fruit size in pot culture and field (Tables 9 and 10). Comparison of the genotypes based on biometric characters is graphically represented in Fig. 3 and Fig. 4. The differences could be attributed to the difference in growth and environmental conditions. Pradeepkumar *et al.* (2001) have reported highly significant differences among tomato cultivars in an evaluation of the cultivars for yield, fruit quality and resistance to bacterial wilt screened under field conditions and pot culture conditions.

Wide variation in plant height was observed among the same genotypes raised in pots and in field. Plant height observed in potted plants was almost





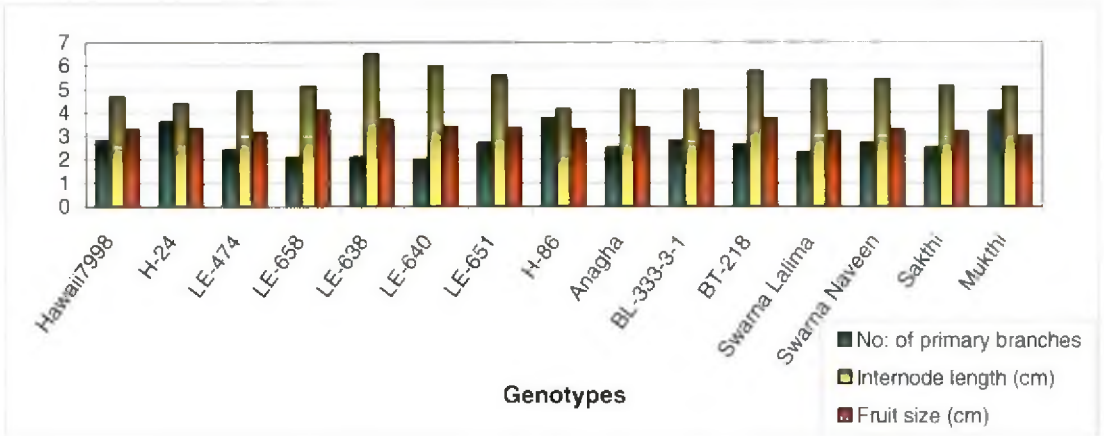
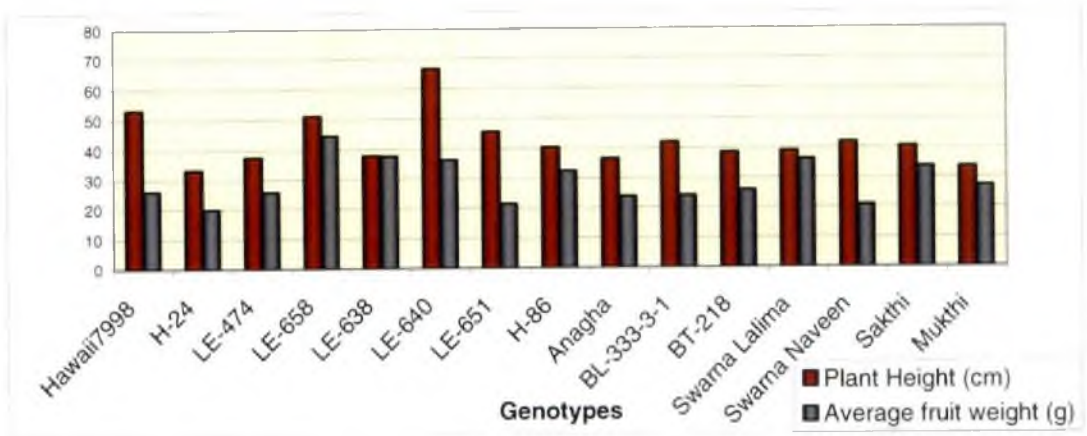
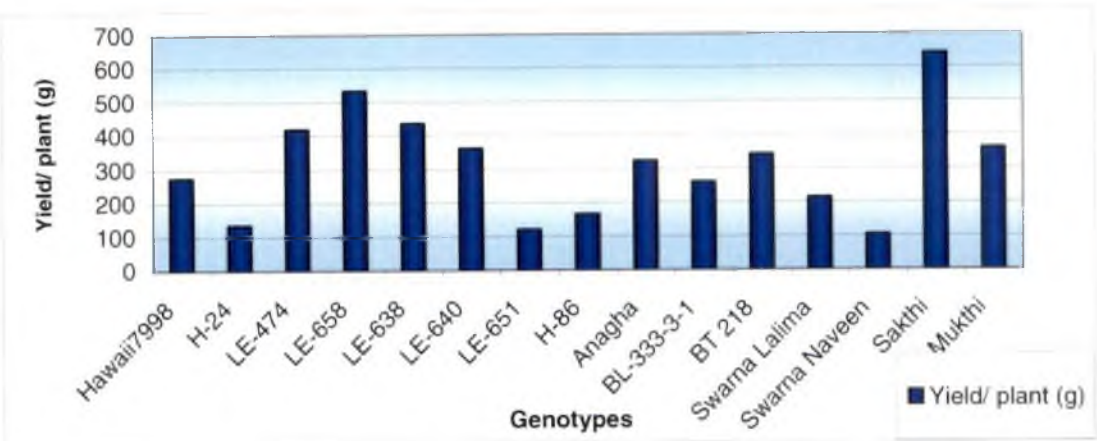


Fig. 3 Biometric characters of tomato genotypes in Pot Culture





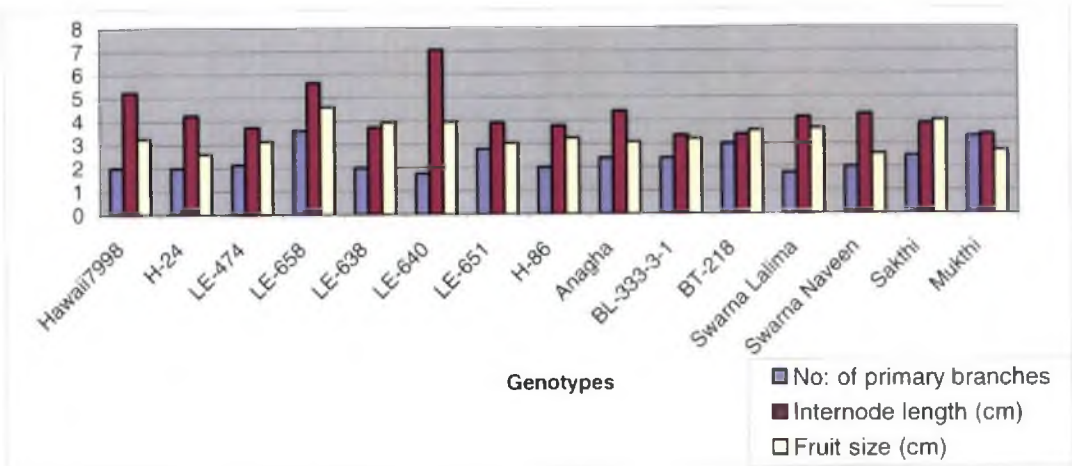


Fig. 4 Biometric characters of tomato genotypes in field condition

double in the case of genotypes like H-24, LE-474, LE-638, LE-640, LE-651, Anagha, BT-218, Swarna Lalima and Swarna Naveen. Lowest plant height observed in the field was 33.3cm in the case of genotype H-24. The reason for taller plants in pots could be better availability of nutrients and water in pot culture. The nutrients in the field are more prone to leaching thereby leading to their non-availability. Same could be the case with field water with the water stress being more pronounced under field conditions due to greater water loss through evaporation. The maximum plant height under both pot culture and field conditions was observed in the plants belonging to the genotype LE-640.

The highest per plant yield was observed in the genotype Swarna Lalima (415.9g) in pot culture and Sakthi (641.5g) in the field (Plate 9), but these genotypes did not show the same kind of domination over the other genotypes with respect to vegetative growth. In the present study, disease symptoms appeared after flowering and during later stages of crop growth. This may have contributed to the high yield obtained from the ToLCV susceptible genotypes Swarna Lalima and Sakthi. Though the genotype LE-640 was superior to the other genotypes with respect to vegetative growth (plant height) both in pot culture and field study, better partitioning of photosynthates to fruit production in the conditions provided might have resulted in higher yield of the genotypes Swarna Lalima and Sakthi. Gururaj *et al.* (2002) have also reported a lack of positive correlation between yield and growth parameters in tomato.

The AVRDC lines included in the study were LE-658, LE-638, LE-640 and LE-651. These lines showed lower per plant yield than the highest yielder Sakthi under field conditions, but all the AVRDC lines excluding LE-651 significantly out-yielded the genotype H-24, considered as a tolerant check to ToLCV, under the same conditions. Similar results were reported with respect to yield of other AVRDC lines and H-24 under field studies conducted at AVRDC, Tawian.

73

5.3 Molecular Characterization

Molecular characterization of the tomato genotypes selected for the study was carried out with the objective of developing trait-related markers for ToLCV resistance/susceptibility. The marker systems used for the study were Random Amplified Polymorphic DNA (RAPD) marker system and Amplified Fragment Length Polymorphism (AFLP) marker system.

5.3.1 Isolation of Genomic DNA

Isolation of good quality DNA is a pre-requisite for RAPD and AFLP assay. Two different genomic DNA isolation protocols reported by two groups of scientists, Doyle and Doyle (1987) and Rogers and Bendich (1994) were tested for suitability of DNA isolation in tomato. DNA isolated by both methods was analyzed by agarose gel electrophoresis using a 0.8 per cent agarose gel. Good quality DNA indicated by discrete bands was obtained by Rogers and Bendich protocol, whereas the DNA isolated by Doyle and Doyle protocol did not appear as discrete bands. RNA contamination was higher in the latter method as compared to the former (Plate 10 and 11). The quantity of DNA obtained by Rogers and Bendich protocol was estimated by UV spectrophotometry. The quantity of DNA isolated ranged from 215 to 255 µg per gram of leaf sample. The ratio of absorbance at 260nm to absorbance at 280nm ranged from 1.78 to 1.88 (Table 11). The value of A_{260}/A_{280} between 1.8 and 2 indicates relatively pure DNA.

DNA was isolated from tender leaves collected early in the morning so as to minimize the interference of polyphenols. Babu (2000) reported that the quality and quantity of DNA isolated was best when tender leaves were used as compared to mature and half mature leaf samples. The use of tender leaves for DNA isolation in tomato has been reported by Martin *et al.* (1991), Archak *et al.* (2002) and Langella *et al.* (2004).

Excess liquid nitrogen was used for homogenization of the leaf tissue, and this could be the reason for obtaining intact DNA. Liquid nitrogen freezes the tissues and helps to prevent degradation of nucleic acid. Similar observations have been made by Sharma *et al.* (2002) in sorghum, chickpea, wheat and soybean, Lodhi *et al.* (1994) in grapevine cultivars and Padmalatha and Prasad (2006) in medicinal and aromatic plants.

The presence of high amounts of contaminating polyphenols was a problem during DNA extraction from tomato leaves. The presence of polyphenols can reduce the yield and purity of DNA by binding covalently with the extracted DNA thus making it useless for most research applications. This problem was overcome by the addition of β-mercaptoethanol and Poly Vinyl Pyrrolidone (PVP) along with the extraction buffer. β-mercaptoethanol disrupts the protein disulfide bonds and is thereby capable of initiating protein degradation. PVP helps removal of phenolic compounds by binding to them with hydrogen bonds and forming a complex. Nesbit *et al.* (1995) and Padmalatha and Prasad (2006) have reported the use of β-mercaptoethanol and PVP for overcoming phenolic contamination.

CTAB (cetyltrimethylammonium bromide), a detergent used in the extraction buffer in Rogers and Bendich protocol has dual functions. On the one hand, CTAB helps in the disruption of the cell membrane thereby releasing nucleic acids into the extraction buffer; while on the other hand, it prevents co-precipitation of polysaccharides with nucleic acid by acting as a selective precipitant of nucleic acids. By these actions, CTAB must have helped in the recovery of relatively pure DNA in the present study. Sharma *et al.* (2002) reported that nucleic acids form tight complexes with polysaccharides creating a gelatinous pellet that contains embedded DNA, and polysaccharides also co-precipitate with DNA after alcohol addition during DNA isolation leading to viscous solutions. Certain polysaccharides are also known to inhibit RAPD reactions. They distort the results and lead to many wrong interpretations

(Padmalatha and Prasad, 2006). So their removal is of importance in DNA isolation.

EDTA present in the extraction buffer protects the DNA from the action of DNase enzyme by chelating and blocking the action of Mg^{2+} ions, which are the major cofactor of DNase enzyme. EDTA is also a major component of TE (Tris EDTA) buffer in which the DNA is dissolved finally. It plays the same role of that of a chelating agent here also.

DNA isolated by Rogers and Bendich (1994) protocol was free from chlorophyll and other pigments. This could be due to the fact that the protocol by Rogers and Bendich involves two treatments with chloroform: isoamyl alcohol (24:1). These treatments ensure the removal of chlorophyll and other colouring substances such as pigments, dyes, etc.

The isolated DNA was found to contain some amount of RNA contamination (Plate 10). Large amounts of RNA in the sample can chelate Mg^{2+} ions and reduce the yield of polymerase chain reaction (PCR). The presence of RNA in the genomic DNA preparation often influences the reproducibility of RAPD patterns (Micheli *et al.*, 1994). In order to overcome the problem of RNA contamination, the extracted DNA samples were treated with RNase A. This treatment yielded intact, discrete DNA bands free from RNA as visualized in agarose gel electrophoresis (Plate 12). The importance of RNase treatment in order to yield RNA-free pure DNA was also reported by Lodhi *et al.* (1994), Archak *et al.* (2002), Archak *et al.* (2003) and Padmalatha and Prasad (2006).

5.3.2 RAPD Assay

Random Amplified Polymorphic DNA (RAPD) assay detects nucleotide sequence polymorphism in a DNA amplification-based assay using only a single primer of arbitrary nucleotide sequence (Tingey and del Tufo, 1993). It is a

dominant marker system that is inherited in a Mendelian fashion (Williams *et al.*, 1990).

In an RAPD reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. A discrete DNA product is formed if these sites are within an amplifiable distance of each other. Each primer will direct the amplification of several discrete loci in the genome thus making the assay an efficient method to screen for nucleotide sequence polymorphisms between individuals.

Random primers obtained from Operon Technologies, USA were used for the study. Operon primer kits are easily available and yield good results. For these reasons they are popular with researchers working on RAPD analysis. The use of Operon primers has been reported by Archak *et al.* (2002) and Rajput *et al.* (2006) in tomato and Clain *et al.* (2004) in *Solanum torvum.*

Out of the 40 random decamer primers belonging to the OPS and OPY series screened initially, 36 primers selected on the basis of robustness of amplification, clarity and scorability of banding patterns were employed for RAPD profiling of the 15 tomato genotypes. The number of amplification products produced by both sets of primers together during primer screening ranged from 2 to 10. This difference in the number of amplification products is due to the fact that even a single base change in the primer sequence could lead to a complete change in the set of DNA fragments amplified as reported by Williams *et al.* (1990).

RAPD profiles for the 15 tomato genotypes were created for each random primer selected after primer screening using the genomic DNA from each of the 15 genotypes as template. Fifteen reactions plus a control were set up for each primer. The control consisted of all the ingredients in the RAPD reaction mixture

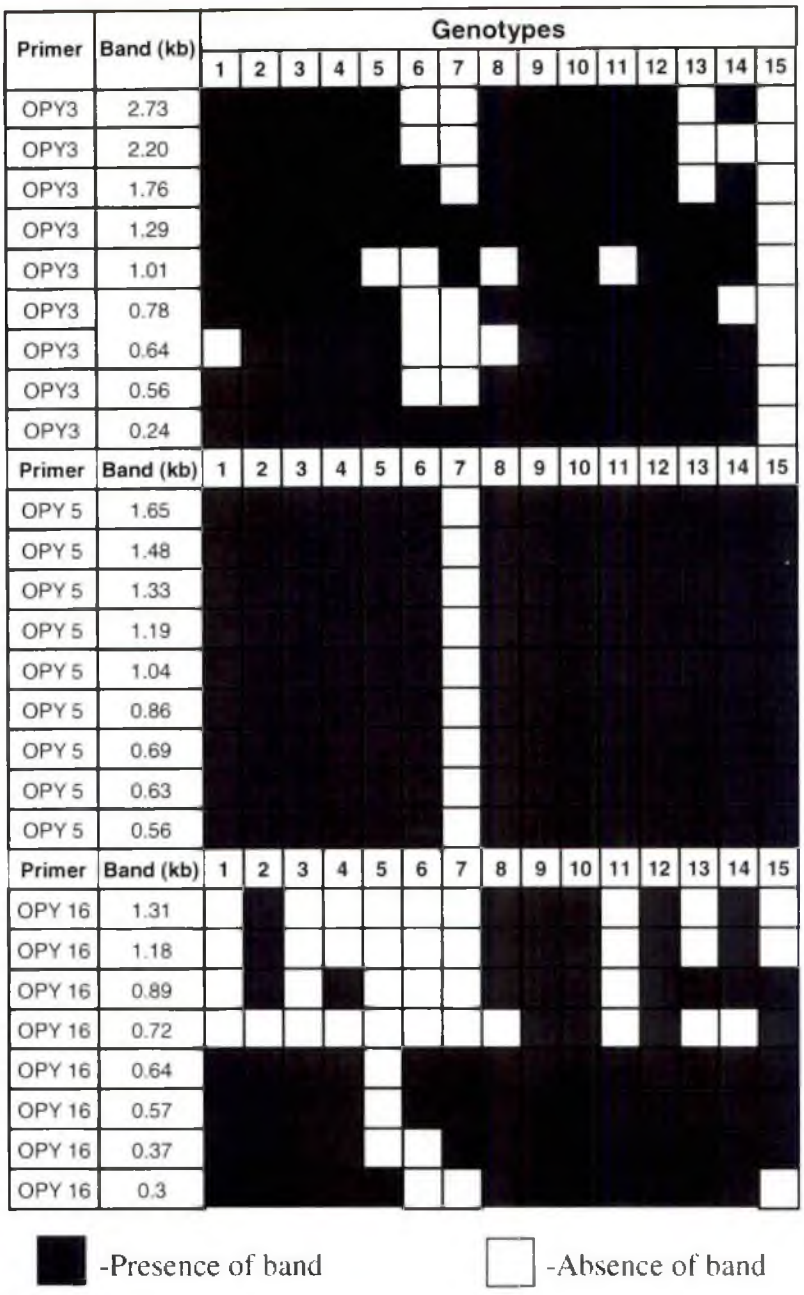
excluding template DNA. The control was maintained in order to ensure that amplification was due to the tomato genomic DNA alone.

Out of the 36 primers selected after primer screening, 12 primers namely OPS 3, OPS 4, OPS 5, OPS 6, OPS 7, OPS 8, OPS 10, OPS 15, OPS 20, OPY 3, OPY 5 and OPY 16 displayed clear and scorable banding patterns after agarose gel electrophoresis with the total number of bands ranging from 5 (OPS 10) to 14 (OPS 3) and size of amplicons ranging from 0.15 kb to 2.88 kb.

For better understanding, pictographs of the banding profiles generated for the 15 genotypes in RAPD assay using the 12 selected primers were prepared with resistant and susceptible genotypes grouped together (Fig. 5, 6, 7 and 8). A total of 116 RAPDs were obtained with the 12 selected primers. The range of polymorphic markers per primer was 0 (OPY 5) to 11 (OPS 3) and the percentage polymorphism ranged from 0 to 80 per cent. The primer OPS 20 gave the maximum polymorphism with eight polymorphic bands out of 10 amplicons. However, the polymorphism observed did not strictly relate to disease reaction in the genotypes.

The primer OPS 8 produced specific bands for the genotypes Mukthi and Sakthi, both being susceptible to ToLCV. This similarity in banding pattern in these genotypes could be due to the fact that the genotype Mukthi was obtained by the process of selection from the genotype Sakthi at the Department of Olericulture, KAU.

Though the percentage polymorphism was as high as 80 per cent, polymorphism was not significant in relation to disease reaction to ToLCV. None of the primers yielded bands specific to more than (or equal to) five resistant or susceptible genotypes.



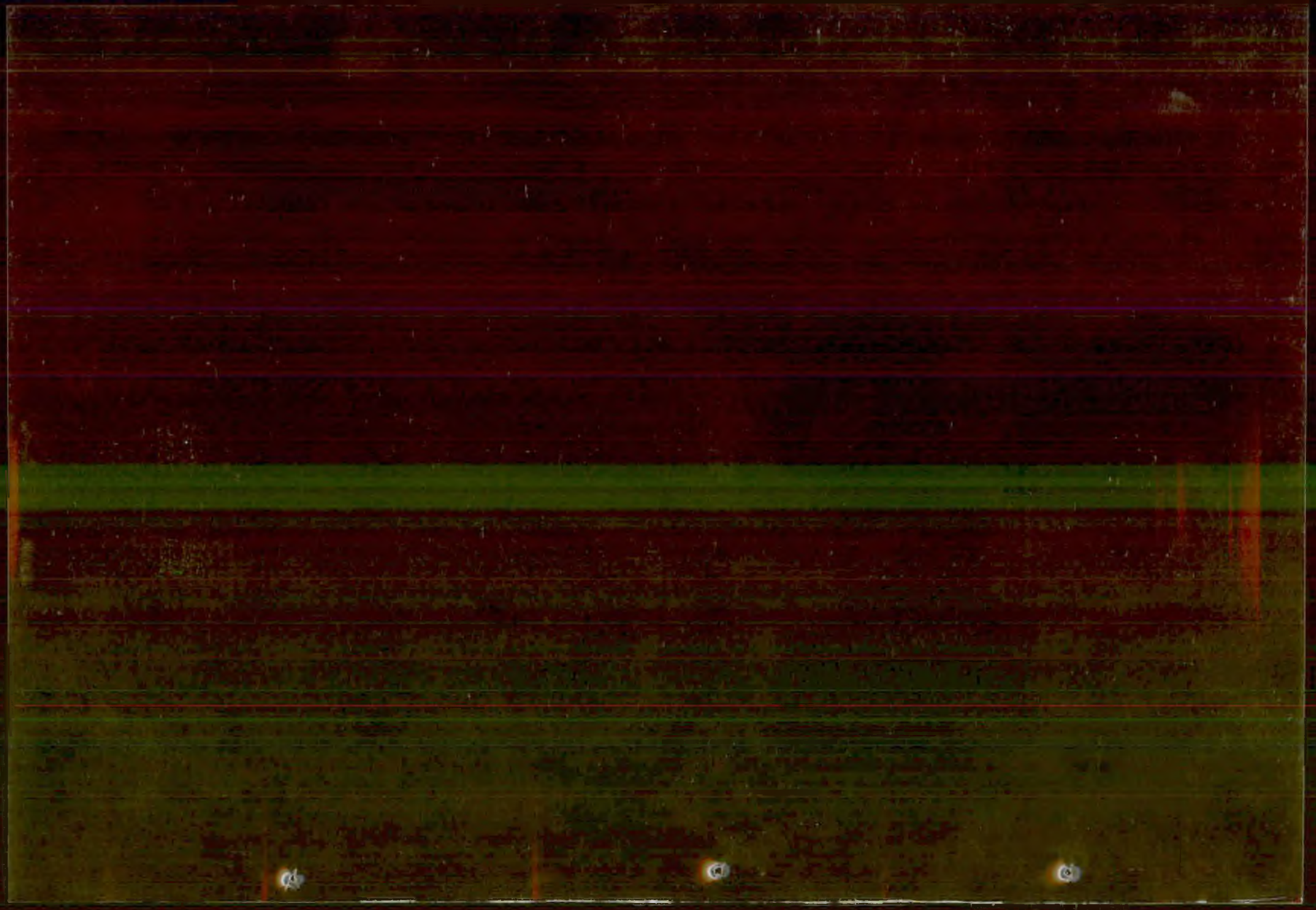
Resistant:

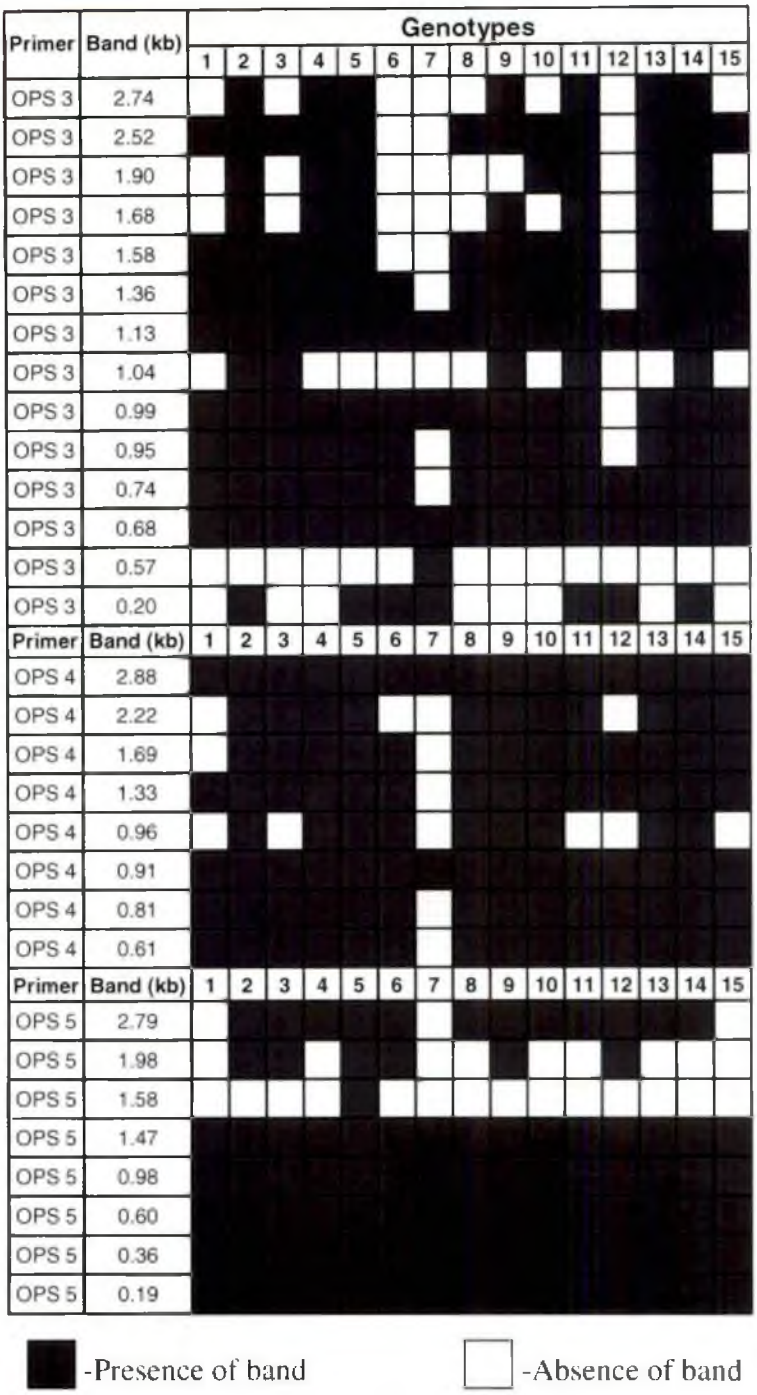
1. Hawaii 7998 **2**. H-24 **3**. LE-474 **4.** H-86 **5.** LE-658 **6.** LE-638 **7.** LE-651 **8.** LE-640 **9.** Anagha

Susceptible:

10. Swarna Lalima **11.** Swarna Naveen **12.** Sakthi **13.** Mukthi **14.** BT-218 **15.** BL-333-3-1

Fig. 5 Pictograph of RAPD profiles generated by primers OPY 3, 5 and 16





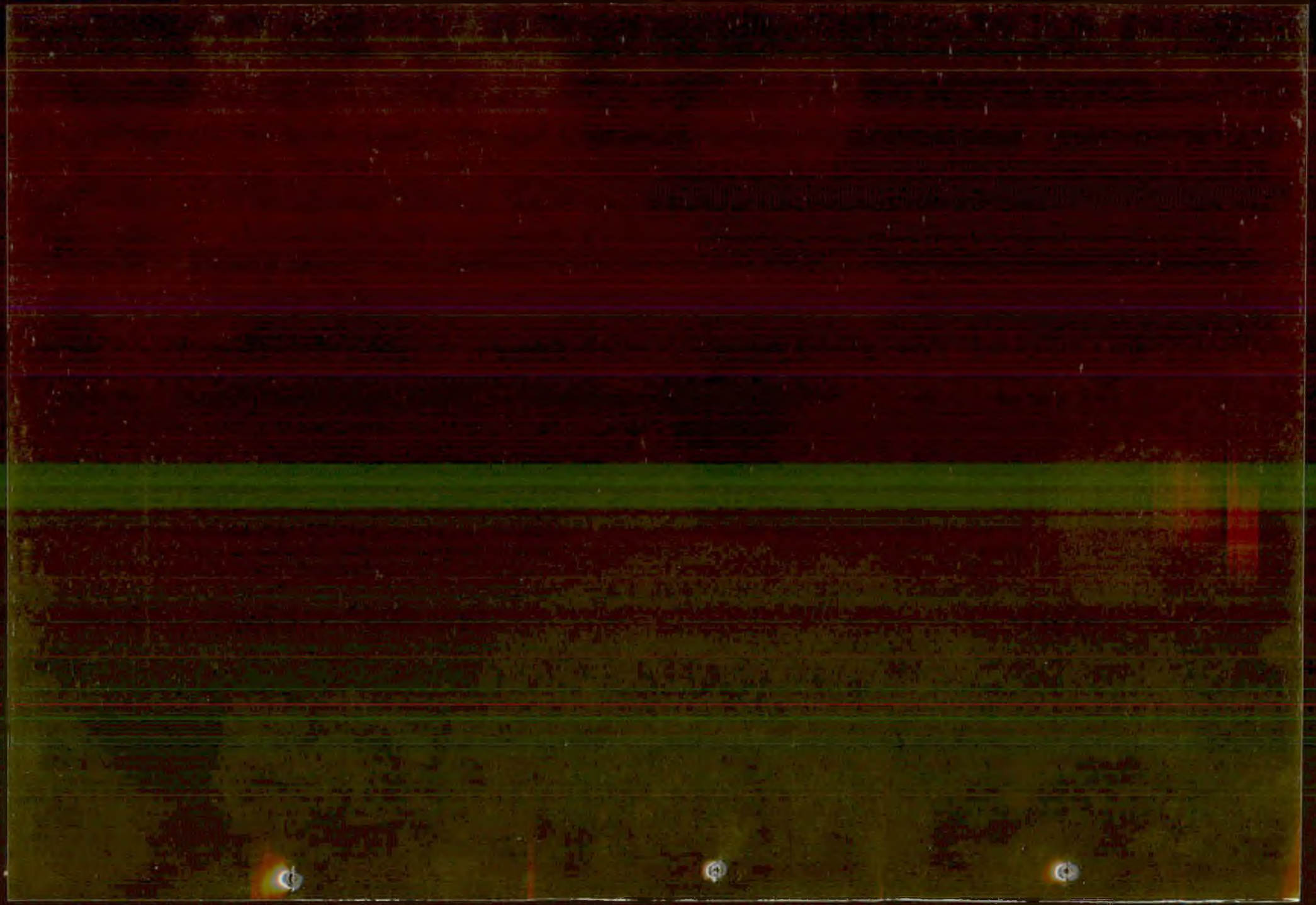
Resistant:

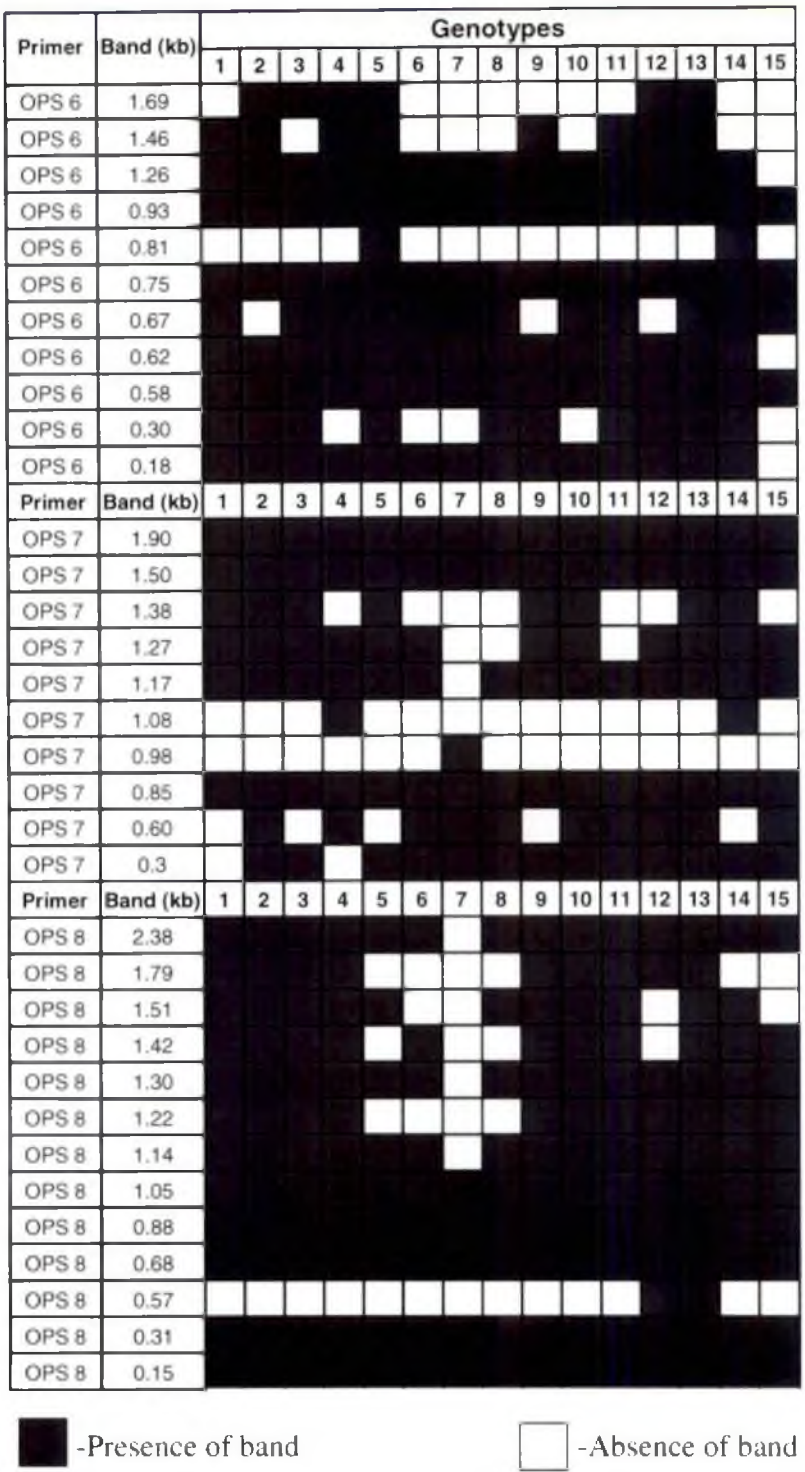
1. Hawaii 7998 **2**. H-24 **3**. LE-474 **4**. H-86 **5**. LE-658 **6**. LE-638 **7**. LE-651 **8**. LE-640 **9**. Anagha

Susceptible:

10. Swarna Lalima **11**. Swarna Naveen **12**. Sakthi **13**. Mukthi **14**. BT-218 **15**. BL-333-3-1

Fig. 6 Pictograph of RAPD profiles generated by primers OPS 3, 4 and 5





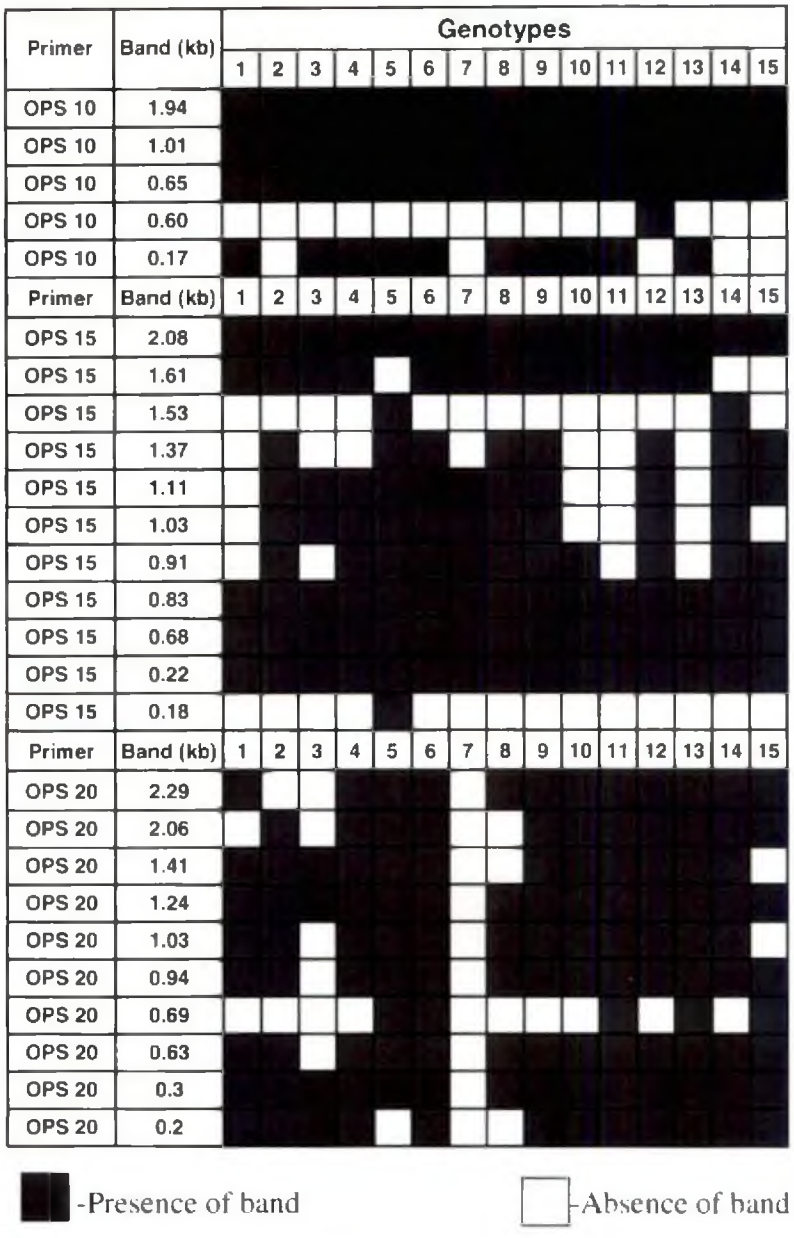
Resistant:

1. Hawaii 7998 **2.** H-24 **3.** LE-474 **4.** H-86 **5.** LE-658 **6.** LE-638 **7.** LE-651 **8.** LE-640 **9.** Anagha

Susceptible

10. Swarna Lalima **11.** Swarna Naveen **12.** Sakthi **13.** Mukthi **14.** BT-218 **15.** BL-333-3-1

Fig. 7 Pictograph of RAPD profiles generated by primers OPS 6, 7 and 8



Resistant:

1. Hawaii 7998 **2.** H-24 **3.** LE-474 **4.** H-86 **5.** LE-658 **6.** LE-638 **7.** LE-651 **8.** LE-640 **9.** Anagha

Susceptible:

10. Swarna Lalima **11.** Swarna Naveen **12.** Sakthi **13.** Mukthi **14.** BT-218 **15.** BL-333-3-1

Fig. 8 Pictograph of RAPD profiles generated by primers OPS 10, 15 and 20

Resolving power (Rp) was calculated for each of the 12 primers selected in the study. Resolving power provides a modest indication of the ability of the primers to distinguish between cultivars (Prevost and Wilkinson, 1999). Resolving power of the primers in the present study ranged from 0.80 to 6.16 with the primer OPS 10 showing the minimum and OPS 3 showing the maximum values. This indicates that the primer OPS 10 was least capable of amplifying the genomic DNA. The use of resolving power to determine the value of primers in RAPD reaction has been reported by Nair (2005).

Amplicons in the RAPD profiles of the 12 selected primers were scored as discrete variables using 1 to indicate presence and 0 to indicate absence. The pair-wise similarity matrix constructed using Jaccard's coefficient indicated great similarity between the genotypes selected for the study (Table 15). Genetic similarity values computed for the 15 tomato genotypes ranged from 0.46 to 0.92.

The dendrogram developed through UPGMA cluster analysis gave a clear picture of relatedness among the genotypes (Fig. 1). No distinct grouping was observed for the susceptible and resistant genotypes studied. However, some of the resistant genotypes were observed to be distinct.

It was interesting to observe the high similarity (92 per cent) between the resistant genotype Anagha released by the Kerala Agricultural University and the resistant genotype H-24 released from IIVR, Varanasi. This could be mainly due to the unique banding pattern observed in these two genotypes using the primer OPS 3 (Fig. 6) (Plate 16(a)). These genotypes were also found to be similar to each other with respect to biometric characters observed in the pot culture study (Table 9) (Fig. 3).

The resistant genotypes Hawaii7998 and LE-474 also formed a single cluster and were distinct from other genotypes. The resistant genotype LE-651 was observed to be unique with only 47 per cent similarity to the other genotypes

and low values of similarity coefficients (Table 15). This type of uniqueness is mainly because of its peculiar amplification pattern observed with the primers OPS 3, OPS 4 (Fig. 6) (Plate 16), OPS 7 and OPS 8 (Fig. 7) (Plate 17 (b) and 17(c)). The plants belonging to LE-651 were also observed to be unique in field evaluation recording poor yield compared to other genotypes.

The AVRDC lines LE-640 and LE-658 were found to belong to the same sub-cluster with them being 79 per cent similar. The AVRDC lines showed a common banding pattern in the amplification profile generated by the primer OPS 8 (Fig. 7) (Plate 17(c)) and this must be the reason for their clustering together in the dendrogram obtained. Such a grouping or uniqueness was not observed for the susceptible genotypes studied.

An overall high level of pair-wise similarity was obtained among the genotypes studied. This could be an indication of the narrow genetic base of the genotypes studied or the poor ability of RAPD analysis to distinguish between tomato genotypes belonging to the same species with respect to ToLCV resistance/susceptibility. The genetic similarity values are reported to be generally high when RAPD markers are used to study polymorphism among accessions within a species (Nair, 2005).

None of the 40 random decamer primers studied could clearly demarcate the resistant genotypes from the susceptible ones. Clear distinction of genotypes based on reaction to ToLCV could not be discerned except in the case of the sub-clusters formed by the genotypes Anagha and H-24, and LE-640, LE-638 and LE-658. The reason for not obtaining ToLCV resistance/susceptibility-related markers in the study could be the higher level of genetic similarity between the selected genotypes. The low level of genetic diversity within cultivated tomatoes has been attributed to self-pollination and artificial selection. Narrow genetic base of Indian tomato cultivars has been reported by Archak *et al.* (2002) through RAPD assay. Chandrashekhara *et al.* (2003) have also reported the high level of

similarity (60 to 84 per cent) in tomato by the use of RAPD analysis. The lack of polymorphism in RAPD analysis between *Solanum torvum* accessions and difficulties for molecular characterization of cultivars in other diploid, autogamous Solanaceae species has been reported by Clain *et al.* (2004). Thus, RAPD markers unique to resistant or susceptible genotypes could not be obtained in the present study.

5.3.3 AFLP Assay

Amplified Fragment Length Polymorphism (AFLP) analysis is a broadly applicable genotyping method with a high degree of reproducibility and discriminating power (Menezes *et al.*, 2003). In AFLPs, the polymorphisms are generated from mutations of the restriction site and (or) between two restriction sites. AFLPs are more sensitive in revealing small mutations than the RAPD technique (Tosti and Negri, 2002).

In the present study, AFLP analysis was carried out using five random combinations of *Eco*RI and *Mse*I based primers. The genomic DNA from 10 genotypes, five resistant and five susceptible, was used as template. *Eco*RI enzyme has an average cutting frequency while *Mse*I has a higher cutting frequency (Savelkoul *et al.*, 1999). A total of 241 bands were detected using the five primer combinations in the present study. Out of the 241 bands detected, 122 were found to be polymorphic. So the percentage polymorphism observed was 50.6 per cent. This level of polymorphism indicates a genetic similarity among the genotypes.

Maximum number of DNA fragments (71) was detected by the combination of EAAC/MCTC based primer pair. The percentage polymorphism was highest by the use of the combination EAAG/MCAC (87 per cent).

In the present study, three markers tentatively linked to ToLCV susceptibility were detected in the susceptible genotypes Sakthi, Mukthi and Swarna Lalima using the primer combination EAAG/MCAC. These genotypes were observed to be susceptible to the disease under both pot culture and field conditions.

The pair-wise similarity matrix constructed on the basis of data obtained by scoring the AFLP profiles generated from the 10 genotypes using five selected primer combinations revealed similarity values ranging from 0.66 to 0.92 (Table 17). Higher similarity values in AFLP assay indicate the high genetic uniformity among the genotypes studied. The genotypes H-24 and H-86 revealed maximum similarity with a similarity coefficient of 0.92. Both these genotypes are highly resistant to ToLCV. These genotypes were obtained from the same source, IIVR, Varanasi and the reason for their similarity in pair-wise analysis also could be that they are derived from a common parental line or due to incorporation of a resistance gene from a common wild species. Both these lines have been reported as good sources of ToLCV resistance and as parents for development of ToLCV resistant hybrids in breeding programs (Kalloo and Banerjee, 2000; Veeraragavathatham *et al.*, 2002; Kumar *et al.*, 2002; Gururaj *et al.*, 2002).

A dendrogram constructed from the similarity data in the present study indicated that all the 10 genotypes selected for AFLP analysis were closely related to each other (Fig. 2). Highly resistant genotypes H-24 and H-86 formed a distinct group with high similarity (93 per cent). The other two resistant genotypes Hawaii 7998 and LE-474 were also grouped together but with less similarity (89 per cent). The resistant genotype Anagha stood as a distinct genotype, but it shared 79 per cent similarity with all the other genotypes except Swarna Lalima. The susceptible genotypes Sakthi and Mukthi having the same origin were found to group together with 91 per cent similarity. The genotype Mukthi was developed from the genotype Sakthi by the process of selection at the Kerala Agricultural University.

Such a grouping was also observed for the susceptible genotypes Swarna Naveen and BL-333-3-1 with 87 per cent similarity. The susceptible genotype Swarna Lalima was distinct from all the other genotypes taken as a group with 26 per cent dissimilarity. This type of separate clustering of all resistant genotypes and all susceptible genotypes was in accordance with the pot culture and field screening data for ToLCV resistance and susceptibility. Thus, AFLP data confirms the narrow genetic base of the selected tomato genotypes initially indicated by RAPD assay in the present study.

However, AFLP analysis revealed distinct clustering of resistant and susceptible genotypes with reference to response to leaf curl caused by ToLCV. AFLP analysis was also helpful in identifying probable markers for ToLCV susceptibility. Three amplicons were observed in the susceptible genotypes with the primer pair EAAG/MCAC (Plate 19), which is indicative of nucleotide sequences that can code for unknown factors responsible for susceptibility. Such factors might favour whitefly attack or may knock down factors responsible for ToLCV resistance in tomato. The nucleotide sequence corresponding to the polymorphic amplicons may be part of a coding sequence, which can code for an inhibitor of the resistance reaction or an inducer of susceptibility. Since the DNA was isolated from healthy young plants, the interference of microbial DNA among the amplicons may be disregarded. The presence of distinct polymorphic amplicons in the susceptible genotypes Sakthi and Mukthi may be due the fact that they are derived from the same centre and they share a common genetic background.

This effectiveness of the AFLP technique compared to other molecular techniques like RAPD, isozymes and RFLP may be due to a more efficient detection of single nucleotide changes at sites for restriction and selective amplification that can be easily resolved and detected in a denaturing poly acrylamide gel under appropriate conditions (He and Prakash, 1997).

The AFLP technique was therefore able to discriminate between ToLCV resistant and susceptible genotypes. This is due to the sensitivity and uniqueness of the regions amplified in AFLP assay. The AFLP assay takes the whole genome into consideration and produces a large number of amplicons, while the RAPD assay does not have genome-wide coverage and amplifies only a limited number of regions of the genome. Resistance to ToLCV has been reported from a number of sources and this resistance could be due to a variety of factors. The grouping of resistant genotypes to different clusters may be due to the origin of resistance inherited from different sources. The polymorphism observed in the present study is unique since none of the resistant genotypes were grouped with the susceptible ones in the dendrogram created. Sequencing of the polymorphic amplicons and studying the expression of these sequences can further unravel the genetic basis of ToLCV resistance/susceptibility in tomato.

Summary

6. SUMMARY

The experiment entitled "Molecular characterization of tomato (*Solanum lycopersicum* L.) with special reference to Tomato Leaf Curl Virus (ToLCV) resistance" was conducted at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, Thrissur with the objective of screening 15 tomato genotypes available at the Department of Olericulture, Vellanikkara for ToLCV resistance at crop level and molecular level under conditions prevalent in Kerala. These genotypes were collected from different centres in India and were already reported to be resistant/susceptible to leaf curl after disease screening at different parts of the country.

The salient findings of the study are summarized in this chapter.

1. The genotypes were screened under natural conditions and scored for their reaction to Tomato Leaf Curl Virus (ToLCV) disease in 35 per cent formaldehyde-sterilized soil in field and pots according to the 0 to 4 score chart suggested by Banerjee and Kalloo (1998).

2. The genotypes Hawaii 7998, H-24, Anagha, H-86, LE-658, LE-638, LE-640 and LE-651 were grouped into the highly resistant category based on the coefficient of incidence values obtained in pot culture conditions. The genotype LE-474 was categorized as resistant to ToLCV under field conditions and highly resistant in pot culture experiment. BL-333-3-1 was found to be moderately susceptible in field and genotypes Mukthi and BT-218 were found to be highly susceptible to the disease under field and pot culture conditions.

3. Highly resistant genotypes were subjected to artificial screening by means of cleft grafting with ToLCV infected scions and were confirmed to be resistant to the disease.

4. Morphological characterization of the 15 tomato genotypes under field conditions and pot culture revealed wide variation in biometric characters like plant height, internode length, number of primary branches, average fruit weight, per plant yield and fruit size.

5. The genotypes were further subjected to DNA based molecular characterization using RAPD and AFLP assays.

6. Genomic DNA was isolated from fresh young tomato leaves collected early in the morning. The protocols suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were tried for DNA isolation. Rogers and Bendich protocol was found to give better quality DNA with lesser RNA contamination.

7. Quantity of DNA isolated by Rogers and Bendich protocol was assessed by spectrophotometric analysis and recovery was found to be high.

8. DNA was treated with RNase A to get pure DNA free from RNA contamination.

9. Forty random primers from two different Operon kits (OPS and OPY) were screened for RAPD assay and out of these, 36 primers with good amplification were selected.

10. RAPD assay of the 15 tomato genotypes was conducted with 36 random primers. Out of these, 12 primers gave better amplification characterized by scorable and clear bands in genotype screening.

11. The total number of RAPD bands generated by all the 12 primers together was 116 out of which 71 bands were polymorphic. Per cent polymorphism ranged from 0 to 80 per cent.

12. Resolving power of the random primers was calculated as per Prevost and Wilkinson (1999). Highest resolving power was calculated for primer OPS 3 (6.16) and least value was calculated for primer OPS 10 (0.80).

13. The RAPD amplification patterns were scored and the data was analyzed using the NTSyS pc. (ver 2.0) software to determine the relationships between the genotypes based on RAPD assay. A dendrogram was prepared based on the similarity coefficients using UPGMA.

14. RAPD assay could not discern any relationship between the genotypes with respect to ToLCV resistance/susceptibility and confirmed the existence of considerable genetic similarity among the genotypes.

15. AFLP assay was conducted using the genomic DNA from 10 selected genotypes as templates. Out of the 10 genotypes used, five were resistant to leaf curl and five were susceptible.

16. The DNA samples were pre-amplified and dilute pre-amplified DNA was used for further analysis.

17. Five different combinations of *Eco*RI and *Mse*I primers were used for AFLP analysis.

18. A total of 241 fragments were detected out of which 122 were polymorphic. The average polymorphism per primer was estimated to be 24.4. Maximum number of bands was obtained using the primer combination EAAC/MCTC. Polymorphism was observed in the profile generated using the primer combination EAAG/MCAC with polymorphic bands appearing in the ToLCV susceptible genotypes.

19. The AFLP profiles generated were scored and data was analyzed using the NTSyS software. Maximum similarity was observed between the genotypes H-24 and H-86.

20. The dendrogram constructed based on the similarity scores revealed distinct clusters of resistant and susceptible genotypes. Though the resistant genotypes were not grouped into a single cluster, there was no mixing of susceptible and resistant ones together in a single cluster.

References

REFERENCES

Abraham, T., Shah, P.S., Rajput, S.G., Anishkumar, P.K., Bansod, S.D. and Tanwar, V.J. 2006. Development of a molecular marker for TLCV resistance in tomatoes [abstract]. In: *Program and Abstracts, International Workshop on Tomato Genomics*, 12- 14, November, 2006, Hyderabad. p.32. Abstract No. 32.

Archak, J.L., Gaikwad, A.B., Gautam, D., Rao, E.V.V.B., Swamy, K.R.M. and Karihaloo, J.L. 2003. DNA fingerprinting of Indian cashew (*Anacardium occidentale* L.) varieties using RAPD and ISSR techniques. *Euphytica.* 230: 397-404.

Archak, J.L., Karihaloo, A. and Jain, A. 2002. RAPD markers reveal narrowing genetic base of Indian tomato cultivars. *Curr. Sci.* 82: 1139-1143.

Babu, T.P.H. 2000. RAPD analysis to assess the genetic stability in tissue culture derived black pepper (*Piper nigrum* L.) plants. M. Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 83p.

Balatero, C.H., Hautea, D.M., Hanson, P.M. and Narciso, J.O. 2002. Development of molecular markers for marker-assisted breeding for bacterial wilt resistance in tomato. *Philipp. agric. Scientist.* 85(2): 170-181.

Banerjee, M.K. and Kalloo, G. 1987. Sources of inheritance of resistance to leaf curl virus in *Lycopersicon. Theor. Appl. Genet.* 73: 707-710.

Banerjee, M.K. and Kalloo, G. 1998. Multiple disease resistant haploid plant of tomato (*Lycopersicon esculentum* Mill.) from anther culture [abstract]. In: *National Symposium on Emerging Scenario of Vegetable Research and Development*, 12-14, December, 1998, Project Directorate of Vegetable Research, Varanasi. p. 93.

Bhattacharjee, I. 1999. Genetics of Yield and its parameters and Resistance to tomato leaf curl virus (ToLCV) in four crosses of tomato (*Lycopersicon esculentum.* Mill). MSc (Ag) thesis, University of Agricultural Sciences, Bangalore, 87p.

Bian, X.Y., Thomas, M.R., Rasheed, M.S., Saeed, M., Hanson, P., De Barro, P.J. Rezaian, M.A. 2007. A recessive allele (*tgr-1*) conditioning tomato resistance to geminivirus infection is associated with impaired viral movement. *Phytopathology.* 97(8): 930-937.

Brar, S.S. and Singh, D. 2003. Evaluation of hot pepper cultivars against leaf curl and mosaic viruses. Proceedings of 43rd and 44th annual meeting. Indian Phytopathological Society, pp. LXXVII-LXXVIII.

Chague, V., Mercier, J.C., Guenard, M., de Courcel, A. and Vedel, F. 1996. Identification and mapping on chromosome 9 of RAPD markers linked to *Sw-5* in tomato by bulked segregant analysis. *Theor. Appl. Genet.* 92: 1045-1051.

Chamber, J.N., Mistry, N.C. and Singh, B. 2006. Indian Horticulture Database- 2005. National Horticulture Board, Gurgaon, 361p.

Chandrashekhara, A.C., Kulkarni, R.S. and Muniyappa, V. 2003. Genetic distance of four species of tomato as revealed by RAPD marker. *Indian J. Genet.* 63(3): 275-276.

Clain, C., Da Silva, D., Fock, I., Vaniet, S., Carmeille, A., Gousset, C., Sihachakr, D., Luisetti, J., Kodja, H. and Besse, P. 2004. RAPD genetic homogeneity and high levels of bacterial wilt tolerance in *Solanum torvum* Sw. (Solanaceae) accessions from Reunion Island. *Plant Sci.* 166: 1533-1540.

Chunwongse, J., Bunn, T.B., Crossman, C., Jian, J. and Tanksley, S.D. 1994. Chromosomal localization and molecular marker tagging of the powdery mildew resistance gene (*Lv*) in tomato. *Theo. Appl. Genet.* 89: 76-79.

Daniel, B. 2003. Screening of tomato somaclones for resistance to ToLCV. M.Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 58p.

Delatte, H., Holotal, H., Reynaud, B. and Dintinger, J. 2006. Chararacterization of a quantitative resistance to vector transmission of tomato yellow leaf curl virus in *Lycopersicon pimpinellifolium*. *Eur. J. Plant Path.* 114(3): 245-253.

Dellaporta, S.L., Wood, J. and Hicks, J.B. 1983. Plant DNA minipreparation: Version II. *Plant Mol. Biol. Rep.* 1: 19-21.

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissues. *Phytochemical Bull.* 19: 11-15.

Engel, M., Fernandez, O., Jeske, H. and Frischmuth, T. 1998. Molecular characterization of a new whitefly transmissible bipartite geminivirus infecting tomato in Panama. *J. General. Virol.* 79(10): 2313-2317.

Foolad, M.R. 2007. Genome mapping and molecular breeding of tomato. *Int. J. Plant Genomics.* 1:1-52.

Gang, G., Yu, Q.D., Yong, L., Ping, J.L. and Xiang, F.L. 2002. A RAPD marker linked to the potato resistance to bacterial wilt. *Acta Phytopathologica Sinica.* 32(3): 267-271.

Gupta, V., Mathur, S., Solanke, A.U., Kumar, R., Vyas, S., Sharma, A.K., Khurana, P., Khurana, J.P. and Tyagi, A.K. 2006. Indian initiative on tomato genome sequencing [abstract]. In: *Program and Abstracts, International Workshop on Tomato Genomics*, 12- 14, November, 2006, Hyderabad. p.33. Abstract No. 33. Abstract No. 33.

Gururaj, Kulkarni, P. and Madalageri, M.B. 2002. Evaluation and correlation studies in tomato for Tomato Leaf Curl Virus [abstract]. In: *Abstracts, International Conference on Vegetables;* 11-14, November, 2002, Bangalore. p.80. Abstract No. II-73-P.

Haanstra, J.P.W., Wye, C., Verbakel, H., Meijer-Dekens, F., van den Berg, P., Odinot, P., van Heusden, A.W., Tanksely, S., Lindhout, P. and Peleman, J. 1999. An integrated RFLP-AFLP map of tomato based on two *Lycopersicon esculentum* x *Lycopersicon pennellii* F_2 populations. *Theo. Appl. Genetics,* 99(1-2): 254-271.

Hanson, P.M., Bernaeehi, S., Green, S., Tanksley, S.D., Muniyappa, V. and Padmaja, A.S. 2000. Mapping of a wild tomato introgression associated with tomato yellow leaf curl virus resistance in a cultivated tomato line. *J. Am. Soc. Hort. Sci.* 125: 15-20.

He, G. and Prakash, C.S. 1997. Identification of polymorphic DNA markers in cultivated peanut (*Arachis hypogaea* L.). *Euphytica.* 97: 143-149.

Hill, S. A. 1984. *Methods in Plant Virology*, Blackwell Scientific Publications, London, 167p.

Hussain, M.A. 1932. Leaf curl in cotton and other plants. *Nature.*3: 130-132.

Kalloo, G. and Banerjee, M.K. 1990. Transfer of tomato leaf curl virus resistance from *Lycopersicon hirsutum f. glabratum* to *Lycopersicon esculentum. Plant Breeding.* 105(2): 156-159.

Kalloo, G. and Banerjee, M.K. 2000. H-24: moderately leaf curl resistant variety of tomato (*Lycopersicon esculentum* Mill.). *Vegetable Sci.* 27(2): 117-120.

Kasrawi, M.A., Suwwan, M.A. and Mansour, A. 1988. Sources of resistance to tomato-yellow-leaf-curl-virus (TYLCV) in *Lycopersicon* species. *Euphytica.* 37(1): 2336-2341.

KAU [Kerala Agricultural University]. 2002. *Package of Practices Recommendations: Crops.* Kerala Agricultural University, Thrissur, pp. 158-159.

Kumar, R., Banerjee, M.K., Verma, N.K. and Chakravorty, S. 2002. Confirmation of resistance against tomato leaf curl virus (TLCV) through grafting in tomato Cv. H-24 [abstract]. In: *Abstracts, International Conference on Vegetables;* 11-14, November, 2002, Bangalore. p.110. Abstract No. II-152-P.

Langella, R., Ercolano, M.R., Monti, L.M., Frusciante, L. and Barone, A. 2004. Molecular marker assisted transfer of resistance to TSWV in tomato elite lines. *J. hort. Sci. Biotech.* 79(5): 806-810.

Lapidot, M. and Friedmann, M. 2002. Breeding for resistance to whitefly-transmitted geminiviruses. *Ann. Appl. Biol.* 140(2): 109-127.

Lodhi, M.A., Ye, G.N., Weeden, N.F. and Reisch, B.I. 1994. A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and Ampelopsis. *Plant Mol. Biol. Rep.* 12(1): 6-13.

Malathi, V.G. 2006. Tomato leaf curl New Delhi virus- the threat is real and spreading [abstract]. In: *Program and Abstracts, International Workshop on Tomato Genomics*, 12- 14, November, 2006, Hyderabad. p.36. Abstract No. 36. Abstract No. 36.

Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D. and Tanksley, S.D. 1993. Map based cloning of a protein kinase gene conferring disease resistance in tomato. *Science.* 262: 1432-1436.

*Martin, G.B., Williams, J.G.K. and Tanksley, S.D. 1991. Rapid identification of markers linked to *Pseudomonas* resistance gene in tomato by using random primers and near isogenic lines. *Proc. Natl. Acad. Sci.,* USA. 88: 2336-2340.

Maruthi, M.N., Alam, S.N., Kader, K.A., Rekha, A.R., Cork, A. and Colvin, J. 2005. Nucleotide sequencing, whitefly transmission, and screening tomato for resistance against two newly described begomoviruses in Bangladesh. *Phytopathology.* 95(12): 1472-1481.

Mejia, L., Teni, R.R., Vidavsky, F., Czosnek, H., Lapidot, M., Nakhla, M.K. and Maxwell, D.P. 2004. Evaluation of tomato germplasm and selection of breeding lines for resistance to begomoviruses in Guatemala. *Acta Hort.* 4: 116-121.

Menezes, C.B., Maluf, W.R., Faria, M.V., Nascimento, I.R. and Stevens, M.R. 2003. AFLP markers linked to spotted wilt virus resistant genes in tomato [abstract]. In: *Abstract, Plant and Animal Genomes XI Conference;* 11-15, January, 2003, California. p.505. Abstract No. 5.1.5.

Micheli, M.R., Bova, R., Pascale, E. and Ambrosio, E.O. 1994. Reproducible DNA fingerprinting with the random amplified polymorphic DNA (RAPD) method. *Nucleic Acids Res.* 22: 1921-1922.

Muniyappa, V., Padmaja, A.S., Venkatesh, H.M., Sharma, A., Chandrashekar, S., Kulkarni, R.S., Hanson, P.M., Chen, J.T., Green, S.K. and Colvin, J. 2002. Tomato leaf curl virus resistant tomato lines TLB111, TLB130 and TLB182. *Hort. Sci.* 37: 603-606.

Nair, S. 2005. Variability in chakkarakolli (*Gymnema sylvestre* R. BR.) using morphological, biochemical and molecular markers. M. Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 125p.

Narasegowda, M.M., Czosnek, H., Vidavski, F. Tarba, S.Y., Milo, J., Leviatov, S., Mallithimmaiah V.H., Seetharam P.A, Subappa, K.R. and Muniyappa, V. 2003. Comparison of resistance to tomato leaf curl virus (India) and tomato yellow leaf curl virus (Israel) among Lycopersicon wild species, breeding lines and hybrids. *European J. Plant Path.* 11: 1-11.

Nesbit, K.A., Potts, B.M., Vaillancourt, R.E., Wests, A.K. and Reid, J.B. 1995. Partitioning and distribution of RAPD variation in a forest tree species, *Eucalyptus globules* (Myrtaceae). *J. Heredity.* 74: 628-637.

Padmalatha, K. and Prasad, M.N.V. 2006. Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from peninsular India. *African J. Biotech.* 5(3): 230-234.

*Pal, B.F. and Tandon, R.N. 1937. Types of tobacco leaf curl in Northern India. *Indian J. agric. Sci.* 7: 368-393.

Pradeepkumar, T., Bastian, D., Joy, M., Radhakrishnan, N.V. and Aipe, K.C. 2001. Genetic variation in tomato for yield and resistance to bacterial wilt. *J. trop. Agric.* 39: 157-158.

Prevost, A. and Wilkinson, M.J. 1999. A new system for comparing PCR primers applied to IISR fingerprinting of potato cultivars. *Theor. Appl. Genet.* 98: 107-112.

*Pruthi, H.S. and Samuel, C.K. 1939. Entomological investigation on leaf curl disease of tobacco in Northern India. *Indian J. agric. Sci.* 11: 389-409.

Rajput, S.G., Wable, K.J., Sharma, K.M., Kubde, P.D. and Mulay, S.A. 2006. Reproducibility testing of RAPD and SSR markers in tomato. *African J. Biotech.* 5(2): 108-112.

Reddy, A.S., Rosaiah, B., Bhaskara Rao, T., Rama Rao, B. and Venugopal Rao, V. 1986. The problem of white fly on cotton. *Proceeding of the seminar on 'Problem of White Fly on Cotton', Pune.* 16th March 1986.

Riccardia, L., Lottib, C., Stefano, P., Yuling, B., Pim, L. and Giovannia, C. 2007. Further isolation of AFLP and LMS markers for the mapping of the Ol-2 locus related to powdery mildew (*Oidium neolycopersici*) resistance in tomato (*Solanum lycopersicum* L.). *Plant Sci.* 172(4): 746-755.

Rogers, S.O. and Bendich, A.J. 1994. Extraction of total cellular DNA from plants, algae and fungi. *Plant Mol. Biol. Manual.* 91: 1-18.

Rohlf, F.J. 1990. NTSYS-PC. Numerical taxonomy and multivariate analysis system. Exeter Software. *Euphytica.* 68: 69-76.

Sadashiva, A.T., Reddy, M.K., Ravishankar, K.V., Reddy, K.M., Singh, T.H., Naveen, L.R., Gunavantha, J., Ravi, B.A., Joshi, S.G. and Mahendrakar, P.

2006. Identification of stable sources of resistance to Tomato Leaf Curl Virus (ToLCV) and development of mapping populations in tomato (*Solanum lycopersicon*) [abstract]. In: *Program and Abstracts, International Workshop on Tomato Genomics*, 12- 14, November, 2006, Hyderabad. p.38. Abstract No. 38.

Saikia, A.K. and Muniyappa, V. 1989. Epidemology and control of tomato leaf curl virus in Southern India. *Trop. Agric.* 66: 350-354.

Saliba-Columbani, V., Causse, M., Gervais, L. and Philouze, J. 2000. Efficiency of RFLP, RAPD and AFLP markers for the construction of an intraspecific map of the tomato genome. *Genome.* 43: 29-40.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual.* Second edition. Cold Spring Harbor Laboratory Press, New York, USA, 1322p.

Savelkoul, P.H.M., Aarts, H.J.M., de Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J.L.W., Schouls, L. and Lenstra, J.A. 1999. Amplified fragment length polymorphism analysis: the state of an art. *J. Clin. Microbiol.* 37(10): 3038-3091.

*Schiewater, B. and Ender, A. 1993. Different thermostable DNA polymerases may amplify different RAPD products. *Nuc. Acids. Res.* 21: 4647-4648.

Seo, Y.S., Gepts, P. and Gilbertson, R.L. 2004. Genetics of resistance to the geminivirus, Bean Dwarf Mosaic Virus, and the role of hypersensitive response in common bean. *Theor. Appl. Genet.* 108: 786-793.

Sharma, A.D., Gill, P.K. and Singh, P. 2002. DNA isolation from dry and fresh samples of polysaccharide-rich plants. *Plant Mol. Biol. Rep.* 20: 415a-415f.

Sharma, T.R. 2003. Molecular diagnosis and application of DNA markers in the management of fungal and bacterial plant diseases. *Indian J. Biotech.* 2: 99-109.

Sinha, S.K., Dasgupta, A., Baranwal, A. and Praveen, S. 2004. Molecular variability in the replicase gene of viruses causing tomato leaf curl disease in India. *J. Plant Biochem. Biotech.* 13(1): 43-46.

Singh, D., Brar, S.S. and Rewal, H.S. 2003. Genetic variability for leaf curl resistance in tomato and selection of resistant cultivars for future breeding programmes. Proceedings of 43rd and 44th annual meeting. Indian Phytopathological Society, pp. LXXXII-LXXXIII.

Smeich, M., Rusinowski, Z., Malepszy, S. and Niemirowicz, S.K. 2000. New RAPD markers of tomato spotted wilt (TSWV) resistance in *Lycopersicon esculentum* Mill. *Acta Physiologiae Pl.* 22(3): 299-303.

Sneath, P.H.A. and Sokal, R.R. 1973. *Principles of Numerical Taxonomy.* W. H. Freeman and Co., San Fransisco, 359p.

Tavella, L., Nervo, G., Accotto, G.P., Vaira, A.M., Acciarri, N., Mason, G., Vecchiati, M., and Schiavi, M. 2005. Field evaluation of tomato hybrids engineered with tomato spotted wilt virus sequences for virus resistance, agronomic performance and pollen-mediated transgene flow. *Phytopathology.* 95(7): 800-807.

Thamburaj, S. and Singh, N. 2001. *Textbook of Vegetables, Tuber Crops and Spice*, Directorate of information and publications of agriculture, IARI, New Delhi, 469p.

Thung, T.H. 1932. De krulen kroepockziekten van tabak en de corzsken van char ver breeding. Proeft. Vor stenlandsche Tabal Meded 72. *Rev. Appl. Mycol.* 11: 478-479.

Tian, M.Y., Feng, L.X., Yang, C.R. and Gong, H.Z. 2000. Identification of a molecular marker linked to ToMV resistance gene *Tm2nv* in tomato using randomly amplified polymorphic DNA. *Acta Phytopathologica Sinica*. 30(2): 158-161.

Tingey, S.V. and del Tufo, J.P. 1993. Genetic analysis with random amplified polymorphic DNA markers. *Plant Physiol.* 101: 349-352.

Tosti, N. and Negri, V. 2002. Efficiency of three PCR-based markers in assessing genetic variation among cowpea (*Vigna unguiculata* subsp. *Unguiculata*) landraces. *Genome*. 45: 268-275.

Tripathi, S. and Varma, A. 2003. Identification of sources of resistance in *Lycopersicon* species to tomato leaf curl geminivirus (ToLCV) by agroinoculation. *Euphytica*. 129(1): 43-52.

Varma, A. and Malathi, V.G. 2003. Emerging geminivirus problems: A serious threat to crop production. *Ann. Appl. Biol.* 142(2): 145-164.

Veeraragavathatham, D., Auxcilla, J., Thiruvudainambi, S. and Muthulakshmi, A. 2002. Breeding tomatoes for leaf curl disease resistance combined with high yield [abstract]. In: *Abstracts, International Conference on Vegetables;* 11-14, November, 2002, Bangalore. p.72. Abstract No. II-51-P.

Vidavsky, F. and Czosnek, H. 1998. Tomato breeding lines resistant and tolerant to tomato yellow leaf curl virus issued from *Lycopersicon hirsutum. Phytopathology*, 88: 910-914.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijter, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23(21): 4407-4414.

Williams, J.G.K., Kubelisk, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.K. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.

Wolf, K., Schoen, E.D. and Peters-Van Rijin, J. 1993. Optimizing the generation of random amplified polymorphic DNAs in chrysanthemum. *Theor. Appl. Genet.* 86: 1033-1037.

Young, N.D., Zamir, D., Ganal, M.W. and Tanksley, S.D. 1988. Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the *Tm-2a* gene in tomato. *Genetics.* 120: 579-585.

Zakay, Y., Navot, N., Zeidan, M., Kedar, N., Rabinowitch, H., Czosnek, H. and Zamir, D. 1991. Screening of *Lycopersicon* accessions for resistance to

tomato yellow leaf curl virus: presence of viral DNA and symptom development. *Plant Disease.* 75(3): 279-281.

Zamir, D., Ekstein-Michelson, I.,Zakay, Y., Navot, N., Zeidan, M., Sarfatti, M., Eshed, Y., Harel, E., Pleban, T., van-Oss, H., Kedar, N., Rabinowitch, H.D. and Czosnek, H. 1994. Mapping and introgression of a tomato yellow leaf curl virus tolerance gene, *TY-1. Theor. Appl. Genet.* 88(2): 141-146.

Zeidan, M., Green, S.K., Maxwell, D.P., Nakhla, M.K. and Czosnek, H. 1998. Molecular analysis of whitefly-transmitted tomato geminiviruses from Southeast and East Asia. *Trop. agric. Res. Extension.* 1(2): 107-115.

* Original not seen.

Appendices

APPENDIX-I

Laboratory Equipments Used For The Study

Spectrophotometer	Spectronic Genesys-5, Spectronic Instrument, USA
Refrigerated centrifuge	Kubota, Japan
Horizontal electrophoresis system	Biorad
Vertical electrophoresis system	Biorad Sequi-Gen® GT sequencing cell
Thermal cycler	1. Eppendorf 2. MJ Research PTC-200 Peltier Thermal Cycler
Gel dryer	Biorad, Model 583
Gel documentation system	1. Alpha Imager 2. Phosphor Imager FLA-5100 Fuji

APPENDIX-II

Composition of Reagents Used for DNA Isolation

1. **Doyle and Doyle method**

   4X Extraction Buffer

   Sorbitol – 2.5g

   Tris HCl – 4.8g

   EDTA – 0.74g

   Dissolved in about 80ml of distilled water, adjusted the pH to 7.5 and made up to 100ml with distilled water.

   Lysis buffer

   Tris HCl (1M, pH 8) – 20ml (15.76g per 100ml)

   EDTA (0.2ml) – 20ml (9.305g per 100ml)

   NaCl (5M) – 40ml (29.22g per 100ml)

   Distilled water – 20ml

   CTAB – 2g (Dissolved in 20ml distilled water and then added to the remaining components).

   5% Sarcosin

   Sarcosin – 5g

   Distilled water – 100ml

   TE Buffer

   10mM Tris (pH 8)

   1mM EDTA (pH 8)

2. **Rogers and Bendich (CTAB) method**

2X CTAB Extraction Buffer

CTAB (2%, v/v)

100mM Tris buffer (pH 8)

20mM EDTA (pH 8)

1.4M NaCl

10% CTAB Solution

10% CTAB (w/v)

0.7M NaCl

TE Buffer

10mM Tris (pH 8)

10mM EDTA (pH 8)

APPENDIX-III

Composition of Buffers and Dyes

1. **TAE Buffer 50X (for 1l)**

   242g Tris base

   57.1ml glacial acetic acid

   100ml 0.5M EDTA (pH 8.0)

2. **TBE Buffer 10X (for 1l)**

   54g Tris base

   27.5g Boric acid

   20ml 0.5M EDTA (pH 8.0)

3. **Loading Dye (6X)**

   0.25% bromophenol blue

   0.25% xylene cyanol

   30% glycerol in water

4. **Formamide Dye**

   Formamide – 10ml

   Xylene cyanol – 10mg

   Bromophenol blue – 10mg

   0.5M EDTA (pH 8.0) - 200µl

APPENDIX – IV

Composition of Reagents used for AFLP Reaction

1. **5X Reaction Buffer**

   50 mM Tris-HCl (pH 7.5)

   50 mM Mg-acetate

   250 mM K-acetate

2. ***Eco*RI/*Mse* I**

   1.25 units/µl each in 10 mM Tris-HCl (pH 7.5)

   50 mM NaCl

   0.1 mM EDTA

   1 mM DTT

   0.1 mg/ml BSA

   50% glycerol (v/v)

   0.1% Triton® X-100

3. **Adapter/ligation Solution**

   *Eco*RI/*Mse* I adapters

   0.4 mM ATP

   10 mM Tris-HCl (pH 7.5)

   10 mM Mg-acetate

   50 mM K-acetate

4. **T4 DNA Ligase**

   1 unit/µl in 10 mM Tris-HCl (pH 7.5)

   1 mM DTT

   50 mM KCl

   50% glycerol (v/v)

5. **TE Buffer**

   10 mM Tris-HCl (pH 8.0)

   0.1 mM EDTA

6. **5X Kinase Buffer**

   350 mM Tris-HCl (pH 7.6)

   50 mM $MgCl_2$

   500 mM KCl

   5 mM 2-mercaptoethanol

7. **T4 Kinase**

   10 units/µl in 50 mM Tris-HCl (pH 7.6)

   25 mM KCl

   1 mM 2-mercaptoethanol

   0.1 µM ATP

   50% glycerol (v/v)

8. **10X PCR Buffer plus Mg**

   200 mM Tris-HCl (pH 8.4)

   15 mM $MgCl_2$

   500 mM KCl

9. ***Eco*RI primers**

   27.8ng/µl

10. ***Mse*I primers**

    6.7ng/µl

    dNTPs

MOLECULAR CHARACTERIZATION OF TOMATO (*Solanum lycopersicum* L.) WITH SPECIAL REFERENCE TO TOMATO LEAF CURL VIRUS (ToLCV) RESISTANCE

By

ANJALI DIVAKARAN
(2005 - 11 - 140)

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture
Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2007

ABSTRACT

Tomato (*Solanum lycopersicum* L.) is one of the major vegetable crops in the world. India ranks sixth in the production of tomatoes worldwide with a total area of 0.50 million hectares and productivity of 17.4 MT per hectare.

Tomato leaf curl disease caused by the Tomato Leaf Curl Virus (ToLCV) and transmitted by whiteflies (*Bemisia tabaci*) is one of the most important diseases affecting this crop. The disease causes losses in yield to the tune of 70 to 100 per cent. ToLCV is severe under conditions prevalent in Kerala also. Identification of resistant sources of the disease and development of trait-related markers from these sources would be an important approach to overcome the problem of ToLCV.

With this objective in mind, an investigation was undertaken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara from the year 2005 to 2007 to characterize the reaction of tomato genotypes to ToLCV under conditions prevalent in the area and to identify molecular markers (RAPD and AFLP) linked to disease resistance.

Fifteen genotypes were raised in sterile soil in earthen pots and field during the peak season of ToLCV infection (December – February) and their reaction to the disease was categorized based on the coefficient of infection. Out of 15 genotypes, eight were observed to be highly resistant to ToLCV under both pot culture and field experiments.

Observations of biometric characters of the genotypes grown in pots and field were made. All genotypes showed significant difference in all the characters observed both in pot culture experiments and field study. Plant height was the most striking character of difference observed in the two different culture conditions.

Genotypes were subjected to molecular characterization using RAPD and AFLP markers. Genomic DNA required for these assays was isolated by two protocols. The protocol suggested by Rogers and Bendich (1994) with modifications was found to be most appropriate for DNA isolation from tomato leaves.

Forty random decamer primers were screened for RAPD assay. Thirty-six of these were used for further RAPD profiling of the tomato genotypes. Out of this, 12 primers displaying good and reproducible patterns were selected for molecular characterization. The primer OPS 8 recorded the highest resolving power. A total of 116 amplicons were generated by the 12 selected primers of which 71 were polymorphic. The dendrogram constructed separated the genotypes into two groups. ToLCV resistant genotypes Anagha and H-24 with 92 per cent similarity were found to be most related. RAPD analysis did not reveal any trait-related marker in the present study.

AFLP assay was carried out with five combinations of *Eco*RI and *Mse*I based primers. A total of 241 amplicons were detected, out of which 122 were polymorphic. Three markers linked to ToLCV susceptibility were obtained using the primer combination EAAG/MCAC.

All genotypes studied showed genetic uniformity in RAPD and AFLP assay except with respect to a few primers. Trait-related marker was detected in a single primer pair in AFLP assay, while RAPD assay did not give any clear demarcation with respect to ToLCV resistance/susceptibility. The markers identified could be further exploited for obtaining nucleotide sequence information and level of specific gene expression in susceptible/resistant genotypes.