MOLECULAR MARKERS FOR BACTERIAL WILT RESISTANCE IN MAPPING POPULATIONS OF TOMATO

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Molecular markers for bacterial wilt resistance in mapping populations of tomato"** 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.

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Certified that the thesis entitled **"Molecular markers for bacterial wilt resistance in mapping populations of tomato"** is a record of research work done independently by Mrs. Ragina.V.C.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.

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Dedicated to My
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF APPENDICES

ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
bp	Base pairs
β	Beta
CPBMB	Centre for Plant Biotechnology and Molecular Biology
cfu	Colony forming unit
^{0}C	Degree Celsius
DAT	Days After Transplanting
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
hr	Hour (s)
KAU	Kerala Agricultural University
kb	Kilo base pairs
L	Litre
Mb	Mega base pairs
M	Mole
ml	Millilitre
mM	Millimole
µg	Microgram
µl	Microlitre
µM	Micromole
ng	Nanogram
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
%	Percentage
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RGA	Resistance Gene Analogues
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
rpm	Rotations per minute
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra Violet
V	Volts
v/v	Volume by volume
w/v	Weight by volume

Introduction

1.INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most popular and widely grown vegetables in the world for it's edible fruits. India is the sixth largest producer of tomato in the world with an area of 0.50 million hectares under cultivation and with a productivity of 17.4 MT/ha (Chamber *et al*. al., 2006). In India, tomato is grown in almost all parts of the country and is used as fresh vegetable, processed and canned products. Apart from vitamin A and C, the fruit also contains lycopene, which has antioxidant properties that reduce risk of some types of cancer and heart diseases.

Tomato cultivation in the tropics is hampered by the incidence of bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. This soil- borne pathogen exists as different races and has a wide host range of over 200 plant species (Buddenhagen *et al*., 1962). Tomato bacterial wilt is mostly caused by Race 1 strains that are highly variable in their genotype and aggressiveness on tomato.

The disease is widespread in most of the tomato growing states of India causing yield losses up to 100 %. Warm humid tropical climate and acidic soil condition in Kerala favour the incidence of the disease. As the application of chemicals, soil fumigation and crop rotation are practically ineffective; use of resistant varieties is the most effective means for control of bacterial wilt.

Molecular mechanism governing bacterial wilt resistance is not well studied. In tomato, resistance to *Ralstonia solanacearum* is polygenic and several loci governing resistance have been reported. It is doubtful whether any single line of tomato exists that carries resistance to all strains of *Ralstonia solanacearum*, since tomato breeding programmes are usually specific for geographic locations and hundreds of different strains of the pathogen are present worldwide.

In disease resistance breeding, breeders frequently counter various interactions among the resistance genes confounding selection through conventional means. These problems can be overcome by identifying molecular markers linked to disease resistance genes (gene tagging) and using these markers for marker assisted selection (MAS).

DNA marker technology has been used in commercial plant breeding programmes since early 1990s. It simplifies the screening for traits that are highly complex provided a closely linked marker to the phenotype is identified. The markers linked to disease resistance can be used for marker assisted selection as it allows rapid identification of plants containing genes of interest, helpful for developing durable resistant genotypes. Apart from that, markers linked to resistance genes may also be useful for cloning and sequencing the gene of interest.

The present study entitled "Molecular markers for bacterial wilt resistance in mapping populations of tomato" was undertaken as part of International SOL Genome Project with an objective to detect and characterize trait related markers with special reference to bacterial wilt.

Review of Literature

2. REVIEW OF LITERATURE

The review of literature on the crop, pathogen causing bacterial wilt, its symptomatology, source of resistance, molecular characterization, bulk segregant analysis of mapping populations, Random Amplified Polymorphic DNA assay, resistant genes and QTL is briefly dealt in this chapter.

2.1. The Crop

The cultivated tomato (*Solanum lycopersicum* L.) is the second most consumed vegetable world wide and a well studied crop species in terms of genetics, genomics and breeding. Tomato belongs to nightshade family solanaceae, which consists of 96 genera and about 2800 species. The cultivated tomato was originally named *Solanum lycopersicum* by Linnaeus. In 1754, Miller separated tomato and designated the genus *Lycopersicon* and the species *esculentum* for the cultivated tomato. More recently based on molecular and morphological information, tomato was renamed as *Solanum lycopersicum* (Majid, 2007).

The cultivated tomato originated in a wild form in the Peru-Ecuador-Bolivia area of Andes i.e. South America. Presently, the genus *Lycopersicon* has been recognized as having nine species, one cultivated and eight wild forms; all having an identical genome 2n = 2x = 24. Cultivated form is *Lycopersicon esculentum* and wild forms are *L. pimpinellifolium, L. cheesmani, L. Chilense, L. hirsutum, L. peruvianum, L. parvifolium, L. chinense, L. penelli* etc. Almost all disease resistance genes in cultivated tomato originated from wild tomato species (Thamburaj and Singh, 2001).

Recent research has revealed that resistance against nearly all serious diseases is available in wild *Lycopersicon* species (Tiwari *et al.*, 2002). Now the

resistant genes from the wild species have been mostly combined in commercial cultivars which can more easily utilized as donor parent (Kalloo, 1986).

The wild species bear a wealth of genetic variability. Less than 10 per cent of the total genetic diversity in the *Lycopersicon* gene pool is found in *L. esculentum* (Miller and Tanksley, 1990).

The cultivated tomato, *Lycopersicon esculentum*, is genetically depleted compared to its wild relatives due to domestication and modern plant breeding (Rick and Chetelat, 1997).

2.2. The Pathogen

Bacterial wilt caused by the soil-borne bacterium *Ralstonia solanacearum* (Smith) Yabuuchi *et al.*, is one of the most destructive plant diseases in the warm humid regions of the world. The pathogen is found to infect a wide range of host plants. The first report about the incidence of disease was from Italy (Walker, 1952).

About one hundred years elapsed since Erwin.F.Smith published the first description of *Pseudomonas solanacearum* E.F.Smith that causes a wilt disease of solanaceous plants (Smith, 1896). Hedayathullah and Saha (1941) first reported the incidence of bacterial wilt disease in tomato from India.

Ralstonia solanacearum is a very complex pathogen, differing in pathogenecity and host range. Geographical variation is seen in the organism. Buddenhagen *et al.*(1962) classified *Pseudomonas solanacearum* isolates from a wide host range of hosts in Central and South America, based on host range, pathogenecity and colony appearance on TZC medium, into 3 races viz. race 1, race 2 and race 3.

1. Race 1 (Solanaceous strain) – It is characterized by wide host range and is seen distributed throughout the lowlands of tropics and subtropics. They attack tomato, tobacco and many solanaceous crops and other weeds.
2. Race 2 (Musaceous strain) – Host range is restricted to *Musa* spp. and a few perennial hosts. Initially limited to American tropics and spreading to Asia.
3. Race 3 (Potato strain) – Host range is restricted to potato and few alternate hosts in tropics and subtropics.

Hayward (1964) classified *Pseudomonas solanacearum* into biotypes or biochemical types namely biotype I, biotype II, biotype III and biotype IV, based on their ability to oxidize various carbon sources and on other bacteriological reactions.

1. Biotype I – doesn't oxidise disaccharides and sugar alcohols.
2. Biotype II – oxidise only disaccharides.
3. Biotype III – oxidises both disaccharides and sugar alcohols.
4. Biotype IV – oxidises only hexahydric alcohols.

Two new races were proposed later, in addition to the first 3 races, one from ornamental ginger as race 4 (Aragaki and Quinon, 1965) and one from mulberry as race 5 (He *et al*., 1983).

A study of thirty tomato isolates of *P. solanacearum* from Assam and Orissa, revealed that all isolates belonged to race 1 (Addy *et al*., 1980).Granada and Sequeira (1983) reported that the *Pseudomonas solanacearum* invades the roots of presumed non hosts such as bean and maize and documented the survival of *P. solanacearum* in the rhizosphere.

He *et al*. (1983) obtained a series of isolates from China which oxidised mannitol but not sorbitol or dulcitol and these were designated as biovar V.Cook and Sequeira (1988) applied RFLP technique to study the relationship between

biovars I to IV of Hayward and races 1, 2 and 3 of Buddenhagen *et al.* They divided *P.solanacearum* into two groups. Strains of race 1, biovars constitute Group I and strains of race 1 biovar 1, race 2 and race 3 constitute Group II. This technique enables the distinction of strains of pathogen with respect to race and biovar. Race 3 strains produced very distinct gel pattern which concluded that race 3 is a homogenous group and fell into three distinct groups representing strains from different geographical origin. In contrast highly variable gel patterns exhibited by race 1 suggested that race 1 is highly heterogenous.

Based on RFLP and other genetic fingerprinting studies, another recent classification of *P. solanacearum* is into Division I which includes biovars III , IV and V originating in Asia and Division II which includes biovars 1, II A and II T, originating in South America (Hayward, 2000).

Kumar *et al.* (1993) classified twelve isolates of *P.solanacaerum* from solanaceous hosts into biovars following Haywards classification. All the isolates from tomato, potato, aubergine and bell pepper (capsicum) were identified as biovar III or a sub type in biovar III. All the isolates utilized glucose, fructose, sucrose, galactose and glycerol.

Yabuuchi *et al.* (1992) transferred several species of the rRNA homology group II *Pseudomonas,* including *Pseudomonas solanacearum* to the genus *Burkholderia*. Sequencing information of 16S rRNA genes and polyphasic taxonomy led to the proposal of genus *Ralstonia* and the pathogen was renamed as *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995).

R. solanacearum pass much of their life cycle in association with their host plants (Allen, 1997). Polymerase chain reaction and amplification with random primers revealed the genetic variation among strains of *R. solanacearum* belonging to race 2 and related bacteria. A transposon induced mutant *R.*

solanacearum strain has lost pathogenecity on its natural host, banana; but is still retaining the ability to wilt tomato (Thwaites *et al.*, 1997).

Paul (1998) identified bacterial wilt affected tomato and chilli isolates as *R. solanacearum* race 1 biovar III. Mathew *et al*. (2000) conducted studies on the isolates of *R. solanacearum* from tomato, brinjal and chilli and identified the pathogen as race 1, biovar III and biovar V.

Variability studies conducted on the isolates of *R. solanacearum* of tomato, brinjal and chilli from different locations of Kerala showed the existence of pathogen belonging to race 1, race 3 and biovar III, III A and V (James, 2001 and Mathew, 2001).

2.3 Symptomatology

First expression of the disease is wilting of lower leaves of the plants and it leads to entire wilting of the plants (Walker, 1952). Dwarfing or stunting may also occur. The entry of the pathogen is through the root system and it was believed that a wound is necessary for the entry (Walker, 1952; Kelman, 1953; Chupp and Sherf, 1960). The entry of the pathogen through natural opening of the plant has been reported by Hildebrandt (1950).

Chupp and Sherf (1960) reported that the bacteria can enter at the points of origin of secondary roots. The roots and lower parts of the stem show a browning of vascular bundles and a water soaked appearance in the root. The pathogen enters into the uninjured roots also (Libman *et al.*, 1964).

Walker (1952) reported that the pathogen first enters into the intercellular spaces of the cortex and from there it moves to pith and xylem vessels leading to vascular plugging and wilting of the plants.

In the advanced stages of infection, dark brown to black areas develop due to decay of root system and the whole plant dies off. A very distinct characteristic indication of bacterial wilt is the appearance of bacterial ooze from the injured vascular regions (Ashrafuzzaman and Islam, 1975).

Break down of plant tissues by the pathogen is due to the cellulose and polygalactouronase enzymes produced by the pathogen. Continued tissue decay and plugging finally result in the death of the plant (Hussain and Kelman, 1957).

Visible symptoms of the disease occur within two to eight days after the entry of the pathogen into the host plant (Kelman, 1953; Chupp and Sherf, 1960). Kelman (1954) noted that virulence might be explained, at least in part by the quantitative differences in EPS (extra cellular polysaccharides) production. The bacterium can produce IAA, which induces the initiation of tylose formation and increases cell wall plasticity.

Sequeira (1993) reported that there is no cytological evidence for how the bacterium reaches the vascular system. It is assumed that the pathogen has to digest its way through the primary wall of the weakened cortical cells as well as of the tracheary elements, where it is exposed between the spiral thickenings.

2.4 Sources of bacterial wilt resistance

Cultivars Louisiana Pink and T-414 from Puerto Rico were found to show good resistance to bacterial wilt, in field trials carried out at North Carolina in USA (Schaub and Baver, 1944). Another source of resistance was reported in *Lycopersicon pimpinellifolium* (PI 127805A), which had partial dominance at seedling stage and the resistance was controlled by recessive genes (Abeygunawardena and Siriwardena, 1963).

Acosta *et al.* (1964) reported that the expression of resistance in a variety is a function of the age of the plant and changes in temperature. Henderson and Jerkins (1972) found that genotypes Venus, Saturn and Beltsville-3814 were resistant to bacterial wilt.

Ahuja and Waite (1974) observed more than 90 per cent survival in BWN-514, BWN-16, BWN-17, and BWN-7755 against *P. solanacearum*. In a diallel test involving six cultivars Graham and Yap (1976) observed that high level of wilt resistance was attained in a breeding procedure of repeated selfing and selection followed by intercrossing of resistant selections. Six cultivars tested for wilt resistance were Walter, CRA 66, H 7741, Venus, VC-4 and Llanos de Coke.

Ramachandran *et al.*(1980) evaluated 36 tomato lines for their resistance to bacterial wilt in Kerala. They observed resistance in La-Bonita and CL-32 d-0-1-19GS.Celine (1981) collected 78 lines to isolate resistant ones and the field screening indicated the tolerance of LE-79, a line from AVRDC, Taiwan.

Tikoo *et al.* (1983) reported the presence of two independent genes for wilt resistance. The resistance was reported to be governed by multiple recessive genes in CRA 66 Sel A from Hawaii and another by single dominant gene in 663-12-3 from Taiwan. Sreelathakumari (1983) reported a complimentary and hypostatic type of digenic recessive gene system for wilt resistance.

Wilt resistance in cultivar Venus and the line CL-32d-0-1-19GS from Taiwan was broken down when *Meloidogyne incognita* larvae were added at the rate of 100/10 cm pot at the time of inoculation with bacteria. The nematode should be considered as a factor in the development of bacterial wilt resistant lines (Goth *et al.*, 1983).

In an experiment to develop new sources of resistance to bacterial wilt, the susceptible check Pusa Ruby showed cent per cent susceptibility (Narayanankutty,

1985). Out of four non segregating lines and two segregating lines evaluated, he observed resistance in F_2 generation of Saturn x LE 79.

Rigorous screening under natural as well as artificial conditions and subsequent selection in the Department of Olericulture, College of Horticulture, Vellanikkara resulted in the development of two bacterial wilt resistant varieties namely Sakthi (LE-79) and Mukthi (LE-79-5).

Rajan and Peter (1986) reported a monogenic incompletely dominant gene action in the resistant line LE-79. Hanudin (1987) reported resistance in cultivars Intan, Ratna, CI 32-6-125-d-0, AV 22 and AV 15. Nirmaladevi (1987) observed that resistance to bacterial wilt in the genotype CRA 66 Sel A was under polygenic control.

Peter *et al.* (1992) reported resistance in the lines LE-214, LE-217, LE-79, LE-70 LFG, LE-79 DG and LE-79 SPF. Evaluation for bacterial wilt resistance by Sadhankumar (1995) revealed consistent resistance of Sakthi and Mukthi. He obtained four additional sources of resistance such as LE-214, CAV-5, LE-415 and LE-382-1.

Chellemi *et al.* (1994) evaluated 30 tomato genotypes for resistance to *R. solanacearum* and observed that the disease incidence ranged from zero in Hawaii 7997, GA 219 and GA 1565 to 83 per cent in Solarset.F_1 hybrids LE 415 x Mukthi, LE 415 x Sakthi, LE 415 x BWR-1 and Sakthi x Mukthi are resistant to bacterial wilt (Rani, 2000).

Evaluation of F_1 hybrids of bacterial wilt tolerant/resistant genotypes Sakthi, LE 214 and LE 206 with HW 208F, St 64, Ohio 8125, TH 318 and fresh market, resulted in the conclusion that these hybrids were completely susceptible to bacterial wilt (Kurian and Peter, 2001).

Sadhankumar (1995) identified a new source of resistance to bacterial wilt in the genotype LE 415.This genotype has an added advantage of resistance to fruit cracking. Later Rajan and Sadhankumar (2002) confirmed the resistance in LE 415 to bacterial wilt. Crop improvement programmes in the Department of Olericulture, Vellanikkara resulted in the release of a wilt and crack resistant line,LE 415 under the name Anagha by the 21 st State Seed Sub committee on crop standards for Kerala(Gokulapalan *et al.*, 2004).

2.5 Mapping population

A mapping population can be derived by selfing the F_1 to produce an F_2, which is then scored for segregation of traits different between parents or by backcrossing the F_1 to one of the parents and observing the segregation in the first back cross generation. It is better to use an F_2 population if it is possible, as more information can be gained from this than a back cross population of comparable size (Chawla, 2002).

The trait to be studied in a mapping population needs to be polymorphic between the parental lines. Additionally, significant trait heritability is essential. It is always advisable to screen a panel of genotypes for their phenotype and to identify the extremes of the phenotypic distribution before choosing the parents of a mapping population.

Phenotypic data of the segregating population, correlated to marker data, prove or disprove potential candidate genes supporting mono and polygenic traits. F_2 populations are the outcome of one meiosis, during which genetic material is recombined. The expected segregation ratio for each codominant marker is 1:2:1 i.e. homozygous like P_1 : heterozygous : homozygous like P_2 (Meksem and Kahl, 2005).

2.5.1.Bulk segregant analysis

Michelmore *et al*. (1991) identified markers linked to a gene conferring resistance to downy mildew in lettuce. They generated bulks of 17 F_2 individuals homozygous for alternate alleles of the resistance locus DM 5/8 and analyzed them with 100 arbitrary RAPD primers to detect around 900 loci. Three RAPD markers linked to the resistance locus were identified. These BSA (Bulk segregant analysis) pools were from the mapping population.

F_2 population derived from a cross between a resistant tomato genotype L 285, and a susceptible cultivar, CLN 286 was analysed using RAPD assay and seven RAPD markers linked to bacterial wilt resistance were added to the map (Aarons *et al*., 1993).

Page *et al*. (1997) used Random amplified polymorphic DNA (RAPD) with the objective of identifying DNA markers linked to sclerotinia crown and stem rot resistance of red clover. Bulked segregant analysis was used to detect polymorphism that should be linked to sclerotinia crown and stem rot resistance. Two bulks were made by pooling previously extracted DNA. Each bulk (one resistant and the other susceptible) consisted of eight genotypes from an F_2 population obtained from a cross between a susceptible and a resistant parent and identified four RAPD markers of sclerotinia crown and stem rot resistance of which three were associated with resistance and one with susceptibility.

Yui *et al*. (1999) obtained four RAPD markers, which are useful for preliminary selection of bacterial wilt resistance, introduced from a bacterial wilt resistant parent Hawaii 7998. F_2 population derived from the cross between susceptible parent Tomato Line 5 (TPL 5) and Hawaii 7998 was screened for these markers. Two markers namely RA 12-13450 and RA 12-291600, were found to be linked to a resistance gene with large effect.

Chen *et al.* (1999) identified two dominant AFLP markers. AF_{348} and AF_{349} linked to Pi44 (t) blast resistance gene using bulk segregant AFLP analysis of F_2 population of RIL 276 (resistant) x CO 39 (susceptible).

AFLP analysis was used in combination with bulked segregant analysis of F_2 mapping population, to identify molecular markers linked to cowpea genes conferring resistance to *Striga geseroioides* race 1.Ouedraego *et al.* (2002) identified seven AFLP markers linked to striga resistance genes in cowpea.

Gore *et al.* (2002) used a population of 1056 F_2 individuals from a cross between soybean mosaic virus and peanut mottle virus resistant line PI 96983 and susceptible cultivar Lee 68 to detect markers RSV 1 and RPV 1 that were linked to soybean mosaic virus and peanut mottle virus resistance.

In an experiment to identify RAPD markers associated with quantitative trait loci for sucrose, using bulked segregant analysis in F_2 population from the melon (*Cucumis melo* L.) cross of TAM Dulce (high sucrose) x TGR 1551 (low sucrose), nine RAPD markers were detected to be significantly associated with QTL for sucrose. In an F_2 population from the different cross of Deltex (high sugars) x TGR 1551 in a field experiment, five RAPD markers were found to consistently associated with QTL for sucrose, total soluble solids and sucrose percentage of total sugars, suggesting that in this cross three sugar traits are controlled by the same QTL (Sinclair *et al.*, 2006).

2.6. Molecular characterization

Molecular biology tools are now being used to facilitate the conventional disease resistance breeding programme and to shorten the duration required to develop a resistant cultivar in different crops (Bent and Yu, 1999).

DNA marker technology has been used in commercial plant breeding programmes since the early 1990s and has proved helpful for the rapid and efficient transfer of useful traits into agronomically desirable varieties and hybrids (Tanksley *et al.*, 1989).

Various PCR based and hybridization based DNA marker techniques can be used for the characterization 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. The tagging of disease resistance genes with molecular markers involves the evaluation of classical phenotype for resistance and molecular marker genotype on the same individuals and the data is analysed to determine, if any of the markers cosegregates with the target phenotype i.e. resistant phenotype. A molecular marker closely linked to a resistance gene can be used for indirect selection of the genes in breeding programme (Sharma, 2003).

2.6.1 Random Amplified Polymorphic DNA (RAPD)

Williams *et al.* (1990) developed a technique that employs random primers in a Polymerase Chain Reaction (PCR) to rapidly generate polymorphic markers that can be used to create genetic linkage maps. They also reported that RAPD is a dominant marker inherited in Mendalian fashion.

Klein-Lankhorst *et al.* (1991) developed a new DNA polymorphism assay based on the amplification by the PCR of random DNA segments, using single primers of arbitrary nucleotide sequence. Original RAPD assay was further increased by combining two primers in a single PCR. They could identify three chromosome 6 specific RAPD markers by comparing the fingerprints of *L. esculentum, L. pennellii* and *L. esculentum* chromosome 6 substitution line LA

1641.One of the RAPD markers was found to be tightly linked to the nematode resistance gene, Mi.

RAPD when used in conjunction with near isogenic lines which differ only by the presence or absence of the target gene and a small region of surrounding DNA, leads to the rapid identification of sequences linked to the gene of interest.

Martin *et al.* (1991) analysed a pair of tomato near isogenic lines that differ for a region on chromosome 5 that contains a gene (Pto) conferring resistance to *Pseudomonas syringae* pv. tomato. He identified the presence of three markers that were generated by random primers to be polymorphic in the Near Isogenic Lines (NILs), and these markers were found to be linked to Pto gene conferring resistance to bacteria *Pseudomonas*.

It has been shown that RAPD markers can vary according to experimental conditions like $MgCl_2$ concentration (Wolf *et al.*, 1993), Taq polymerase quality and quantity (Schiewater and Ender, 1993) and type of thermal cycler (Wolf *et al.*, 1993).

Chunwongse *et al.* (1994) reported the chromosomal localization and molecular tagging of the powdery mildew resistance gene (Lv) in tomato using RAPD and RFLP markers. DNA from a resistance and a susceptible cultivar were screened with 300 random primers that were used to amplify DNA of resistant and susceptible plants. Four primers yielded fragments that were unique to the resistant line and linked to the resistance gene in an F_2 population.

Bulked segregant analysis was used to identify RAPD markers linked to the Sw-5 gene for resistance to tomato spotted wilt virus in tomato (Chague *et al.*, 1996). Using two pools of phenotyped individuals from one segregating

population, they identified four RAPD markers linked to Sw-5 gene. They have reported that two of these markers, R_2 and S_1, are tightly linked to this gene.

Gang *et al.* (2002) reported polymorphic markers between resistant bulk and susceptible bulk DNA of *Solanum phureja* by RAPD analysis with 300 random primers. The primer OGP 09 produced a 960bp reproducible band only in resistant clones, linking to the wilt resistance in the population.

Low levels of polymorphism were reported using RAPDs in tomato (Archak *et al.*, 2002). Difficulties for molecular characterization of cultivars in other diploid autogamous solanaceae species are reported.

The homogeneity between accessions of *Solanum torvum* regarding high bacterial wilt tolerance was confirmed by the genetic homogeneity (zero per cent polymorphism) revealed using 168 RAPD primers.Clain *et al.* (2004) reported *S. torvum* as a potential source of bacterial wilt disease for cultivated solanaceae species.

2.7. RESISTANCE GENES (*R* genes) and QTL (Quantitative Trait Loci)

Many plant–pathogen interactions are determined by the presence or absence of complementary pairs of resistance (R) genes in the host and avirulence genes (*avr*) in the invading microorganisms. In the elicitor–receptor model proposed to account for this gene-for-gene theory (Flor, 1971), *avr* genes encode elicitors that serve as ligands for receptors encoded by *R* genes, which trigger a complex defense response.

Several quantitative trait loci (QTL) have been shown to play a role in resistance to bacterial wilt in different studies using different populations and at different geographic locations (Danesh *et al.*, 1994).Many of these *R* gene products share structural motifs, which indicate that resistance to diverse pathogen

may operate through similar pathways. The use of molecular markers and interval mapping is a powerful approach that permits the identification and genetic mapping of loci controlling a trait of interest.

Martin *et al.* (1992) reported that resistance to *Pseudomonas syringae* pv *tomato* (*Pst*) is conferred by a single dominant locus, *Pto*. They identified RFLP and RAPD markers that are tightly linked to this locus in near isogenic lines of tomato. Multistep positional cloning strategy was used to isolate the *Pto* locus.

Later, Martin *et al.* (1993) constructed a high-resolution linkage map containing 18 molecular markers and spanning 20 centi Morgans of tomato chromosome 5 for the region containing *Pto*. One marker identified, TG 538, co-segregated with *Pto* and therefore provides a starting point for chromosome walking. This gene code for Serine / Threonine rich protein kinase conferring resistance to *Psuedomonas syringae* pv *tomato*.

Thouquet *et al.* (1996) developed a molecular map of F_2 population of Hawaii 7996 X Wva 700 using RFLP. Nine independent resistance tests were conducted and identified that the most important QTL was located on chromosome 6, and another QTL on chromosome 4 and a weaker putative QTL at other map positions.

Deberdta *et al.* (1999) showed that the presence of the *Mi* gene was associated with a marked decrease in bacterial wilt resistance. They suggested that at least one gene governing part of the bacterial wilt resistance is closely linked or allelic to the *Mi* gene in the tomato lines Caraibo, CRA66 and Cranita.

Deslandes *et al.* (2003) did the identification and mapping of RRS1, a Single Recessive Locus in *Arabidopsis thaliana* (Nd-1 ecotype) that confers resistance to *R. solanacearum*. *RRS1-R* is the first characterized *R* gene conferring resistance to *R. solanacearum*, and its characterization, the elucidation of its mode

of action, as well as its intergeneric transfer in various crops are important steps in facilitating the elaboration of new approaches to disease control.

Chi *et al*. (2004) introduced the Arabidopsis *NPR 1* (nonexpressor of PR genes) gene into a tomato cultivar, which possesses heat tolerance and resistance to tomato mosaic virus. Disease screens against eight important tropical diseases revealed that, in addition to innate ToMV resistance, the tested transgenic lines conferred significant level of enhanced resistance to bacterial wilt and *Fusarium* wilt.

Materials and Methods

3. MATERIALS AND METHODS

The detection and characterization of trait related markers for bacterial wilt resistance in mapping populations of tomato was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Olericulture, College of Horticulture, Vellanikkara during the period 2006 to 2008. Methodology and materials used for the study has been described in this chapter.

3.1 MATERIALS

3.1.1 Plant material

Identified bacterial wilt resistant variety Anagha, released from Kerala Agriculture University was used as the resistant parent. DVRT-1 and Pusa Ruby were used as the susceptible parents (Table 1).

Table 1. Source and reaction of tomato genotypes to bacterial wilt

Variety	Source	Character specific to bacterial wilt
Anagha	KAU, Thrissur	Resistant to bacterial wilt
Pusa Ruby	IARI, New Delhi	Susceptible to bacterial wilt
DVRT-1	IIVR, Varanasi	Susceptible to bacterial wilt

3.1.2 Chemical, glassware and plastic ware

All the chemicals used for DNA isolation and RAPD were procured from the companies MERCK, SRL and HIMEDIA in India. The kit used for DNA isolation was from Chromous Biotech Pvt. Ltd.Banglore and molecular biology grade enzymes and buffers were from Bangalore Genei Ltd. The primers were provided by Operon Technology, USA .All the plastic wares used were obtained from Tarsons Products Pvt. Ltd.

3.1.3 Equipment and machinery

The equipment items like centrifuge (KUBOTA 6000), PCR machine (Eppendorf), horizontal gel electrophoresis system (BIORAD), oven etc; available at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture were used for study. Gels were documented using UVP GelDoc- It ^{TM} Imaging system (UVP, UK).

3.2 METHODS

3.2.1 PRODUCTION OF MAPPING POPULATIONS OF TOMATO

A) Raising the parents

The three selected tomato varieties viz. Anagha, Pusa Ruby and DVRT-1 were grown during November, 2006 to February 2007. The seeds were sown in pots containing sterilized 1:1:1 mixture of sand, soil and FYM. Before sowing the seeds, the medium was sterilized using formaldehyde solution (1:30).The solution was applied to the potting mixture in pots and covered with polythene sheets. After one week, the polythene sheets were removed and the pots were kept open for another one week to remove the residual effect of formalin. Seeds were sown in these pots and maintained in the nursery for 30 days.

Seedlings of Anagha were transplanted to a bacterial wilt sick plot, after one month. Seedlings of Pusa Ruby and DVRT-1 were transplanted to pots filled with sterilized medium.

B) Production of F_1 generation by crossing parents

F_1 populations were developed by crossing the parents. Two crosses were made i.e., Anagha x Pusa Ruby and Anagha x DVRT-I. For crossing, flowers of Anagha were emasculated at 3 pm on the day prior to anthesis and covered with

butter paper cover. Those flowers emasculated were pollinated on the next day morning at 8 am, using the fresh stamen collected either from flowers of Pusa Ruby or DVRT-I. The pollinated flowers were covered and tagged indicating the cross and date of pollination.

After one week, the butter paper covers were removed. These fruits were harvested at red ripe stage and seeds were extracted. The F_1 plants were tested for resistance in a bacterial wilt sick field (having a population of 1.8-6.3 $x10^8$ cfu per g of soil).The plot size was 3mx3m and spacing was 60cm x 60cm.There were three replications.

C) Production of F_2 population

Seedlings of F_1 population were also raised in pots filled with sterilized medium and kept in green house. Management practices were followed as per KAU package of practices recommendations (2007).F_1 plants were selfed to produce F_2 seeds.

D) Raising the F_2 population in bacterial wilt sick plot

Two hundred seeds of F_2 population of both the crosses were sown in protrays with sterilized vermicompost (Plate 1). The individual units of protrays were numbered from one to two hundred for both the crosses. One month after sowing, seedlings were transplanted to bacterial wilt sick plot according to the number given to the plants and were tagged individually indicating the cross and the number. Management practices were followed as per KAU package of practices recommendations (2007).

The transplanted F_2 populations were observed daily for the development of disease symptoms. Bacterial wilt is characterized by sudden complete wilting of the plant. The disease infection was confirmed by ooze test. The wilted plants

Plate 1.Protray or seedling tray used for raising the nursery of F_2 population

were uprooted, washed under tap water and stem portion just above root system was cut and the cut end was placed in a test tube containing clear water and allowed to stand for few minutes. The streaming of whitish ooze from the cut end confirmed the wilt infection.

Based on the observation on wilt incidence, the percent wilt incidence was calculated using the formula.

$$\text{Percent bacterial wilt incidence} = \frac{\text{No. of plants wilted}}{\text{Total no. of plants observed}} \times 100$$

3.2.2 MOLECULAR CHARACTERIZATION

3.2.2.1 Isolation of genomic DNA

Genomic DNA was isolated from parents, F_1 and F_2 population of both the crosses for RAPD analysis. The leaf samples collected in the morning from young seedlings were ground individually in liquid nitrogen and the powder was covered by Aluminium foil, tagged ,preserved at -80 ^{0}C until further use for DNA isolation. The DNA from such samples was isolated using Rogers and Bendich method (1994) and the plant genomic DNA isolation kit from Chromous Biotech Pvt. Ltd.

3.2.2.1a) DNA isolation method by Rogers and Bendich protocol (1994)

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

Reagents used

- 2 x CTAB extraction buffer
- 10% CTAB solution
- TE buffer

- Isopropanol
- Chloroform: Isoamyl alcohol mixture (24:1 v/v)
- Ethanol (100% and 70%)

The details of preparation of reagents are provided in Appendix-I.

Procedure

- Leaf tissue (1 g) kept at $^{-}80\ ^{0}C$ was ground in excess liquid nitrogen and 4 ml of 2x extraction buffer and 100 µl β-mercapto ethanol were added
- The ground tissue was transferred into a 50 ml oakridge tube containing 3 ml prewarmed extraction buffer. The contents were mixed well and incubated at 65°C for 15 minutes
- Equal volume of chloroform: isoamyl alcohol mixture was added, mixed gently by inversion and centrifuged at 10,000 rpm for 10 minutes at 4°C
- The upper aqueous phase containing DNA was transferred to a fresh 50 ml oak ridge tube and $1/10^{th}$ volume of 10% 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
- The upper aqueous phase was collected in a fresh oak ridge tube and 0.6 volume of chilled isopropanol 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
- The 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, resuspended in 100 µl TE buffer and stored at -20°C until further use

3.2.2.1. b. DNA isolation by plant genomic DNA spin kit from chromous Biotech Pvt. Ltd.

Kit components

- Plant gDNA Suspension Buffer (5x)
- Plant gDNA Lysis Buffer (1x)
- RNase A solution DNase free (10 mg/ml)
- Wash Buffer : 10 ml concentrate
- Elution Buffer : 5 ml
- Spin columns

Protocol

- Hundred mg of ground leaf tissue kept at -80 ^{0}C was taken in a mortar and pestle
- Added 750 µl of suspension buffer; ground the leaf tissue, until it form a fine paste. Pipetted this into a 2 ml vial (using a tip that is cut at the bottom).
- To the above added 5 µl of RNase solution provided. Mixed 5-6 times by inverting the vial. Placed it at 65°C for 10 minutes with intermittent mixing
- To the above added 1 ml lysis buffer and mixed 5-6 times. Kept the mixture at 65°C for 15 minutes. Cooled it to room temperature
- Spun at 13,000 rpm for 1 minute at room temperature and collected the clear supernatant in a 2 ml vial
- Loaded the supernatant on the spin column (600 µl each time)
- Spun at 13,000 rpm for 1 minute at room temperature. Discarded the contents of the collection tube. Placed the spin column black in the same collection tube
- Added 500 µl of Wash Buffer to the column. Span at 13,000 rpm for 1 minute at room temperature. Discarded the contents of the collection tube. Placed the spin column back in the same collection tube.

- Repeated step 8
- Spun the empty column with the collection tube at 13,000 rpm for 2 minutes at room temperature
- Placed the spin column in a fresh vial
- Added 40 µl of Warm Elution Buffer (already kept at 65°C) into the spin column
- Kept the vial along with the spin column at 65°C for 1 minute. Spun at 13,000 rpm for 1 minute at room temperature
- Repeated step 12 and 13. Eluted and collected in the same vial

3.2.2.2 Purification of DNA

The DNA isolated by Rogers and Bendich protocol was purified from RNA contamination by RNase treatment (Sambrook *et al.*, 1989).

Reagents used

- Phenol: Chloroform mixture (1:1, v/v)
- Chilled isopropanol
- Ethanol (70%)
- TE buffer
- Chloroform: Isoamyl alcohol (24:1; v/v)
- RNase

Procedure

- RNase solution (2 µl) was added to 100 µl DNA sample and incubated at 37°C in dry bath for 1 hour
- The volume was made up to 250 µl with distilled water and equal volume of phenol chloroform mixture was added
- Centrifuged at 12,000 xg for 10 minutes at 4°C
- Collected the aqueous phase in a fresh micro centrifuge tube and added equal volume of chloroform : Isoamyl alcohol (24:1)

- Centrifuged at 12,000 g for 10 minutes at 4°C
- The above two steps were repeated and finally precipitated DNA from the aqueous phase with 0.6 volume of chilled isopropanol
- Incubated the mixture at -20°C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4°C
- The pellet of DNA was washed with 70 per cent ethanol
- Air dried the pellets and resuspended in 100 µl TE buffer and stored at -20°C until further use
- Five microlitre of this purified DNA was run on agarose gel to check for complete removal of RNA

3.2.2.3 Estimation of Quality of DNA

The quality of isolated DNA was determined through agarose gel electrophoresis (Sambrook *et al.*, 1989).

Materials for agarose gel electrophoresis

- Agarose (Genei, Low EEO) - 0.7 per cent (for genomic DNA)
  - 1.2 per cent (for RAPD)

- 50 x TAE buffer
- 6 x Loading / Tracking dye
- Ethidium Bromide solution (stock 10 mg/ml; working concentration: 05 µl/ml)
- UV transilluminator (Herolab^{R})
- Electrophoresis unit, power pack, gel casting tray, comb
- Gel documentation and analysis system (UVP GelDoc- It ^{TM} Imaging system (UVP, UK)

Composition of reagents is provided in Appendix-II.

Procedure

- The 50x stock solution of TAE buffer was diluted to1x concentration of required quantity according to the capacity of gel casting tray
- The required quantity of agarose was weighed, added to the 1x TAE buffer and melted completely by boiling
- The solution was cooled to luke warm temperature (55°C) the ethidium bromide was added at a concentration of 0.5 µg/ml as an intercalating dye of DNA, which will help in its visualization in UV rays and mixed well
- The open ends of gel casting tray were closed tightly by placing the casting tray in and tightening with the screw
- The comb was placed properly (i.e. the comb should be 5 mm above the casting tray) and the agarose solution was poured into the gel casting tray
- After 30 minutes the comb was removed carefully by pulling and the gel along with casting tray was placed in electrophoresis unit containing 1x TAE buffer with the wells directed towards the cathode. Required quantity of 1x TAE buffer was added so as to submerge the gel to a depth of 1 cm
- 5 µl of DNA was mixed with 1 µl of 6x loading dye and loaded into the wells using micro pipette. The marker used was λ DNA / *EcoR I* + *Hind III* double digest (Bangalore Genei) and it loaded in first well after mixing with loading dye
- The power supply was turned on a feel checking the connection of anode and cathode to electrophoresis unit. The gel was run at constant voltage (100 volts)
- When the tracking dye reached 2/3rd length of the gel, the current was disconnected and the gel was taken from electrophoresis unit

- The gel was placed in a gel documentation system (UVP GelDoc- It ™ Imaging system (UVP, UK) and bands were visualized under UV light. The quality of DNA extracted was ensured

3.2.2.4 QUANTIFICATION OF DNA

Quantity of the DNA isolated was analyzed using nanodrop spectrophotometer. The Nanodrop ND-100 is a full spectrum (220-750 nm) spectrophotometer that measures 1 µl sample with high accuracy and reproducibility.
General operation of spectrophotometer is as follows.

1) With the sampling arm in open position, pipetted the sample (1 µl DNA) on to the lower measurement pedestrals

2) Closed the sampling arm and initiated the spectral measurement using the operating software in the computer. The sample column was automatically drawn between the upper and lower measurement pedestrals and the spectral measurement was made

3) When the measurement was complete, opened the sampling arm and wiped out the sample from both pedestrals using tissue paper to prevent sample carry over in successive measurements

3.2.2.5 RAPD (Random Amplified Polymorphic DNA Assay)

RAPD analysis was performed for characterizing the isolated DNA from parents, F_1 and F_2 population of both the crosses. Random Amplified Polymorphic DNA (RAPD) assay is a PCR based molecular marker system that uses arbitrarily selected decamer primers to amplify a set of DNA fragments that are located at random positions in the genome. The ten nucleotides long primers can be

annealed at a number of locations in the genome giving a number of amplification products. The product of RAPD assay was separated on 1.2 per cent agarose gel. Genomic DNA from different genotypes or individuals often produces different amplification patterns. A particular fragment generated for one individual, but not for other represents DNA polymorphism and can be used as a genetic marker.

Different constituents of an RAPD assay reaction mixture are DNA, random primer, enzyme, dNTPs, $MgCl_2$ and Taq buffer etc. These constituents are subjected to repeated cycles of denaturation, primer annealing and extension in a thermal cycler to produce amplification.

3.2.2.5.1 Protocol for RAPD assay

RAPD assay was carried out in a thermal cycler (Mastercycler personal, Eppendorf) using the procedure suggested by Williams *et al*. (1990), with some modifications. The programme was done in heated lid condition. Protocol for tomato genome include

Initial denaturation – 94°C for 5 minutes
Denaturation – 94°C for 1 minute
Primer annealing – 37°C for 1 minute
Primer extension – 72°C for 2 minutes
Final extension – 72°C for 2 minutes
4°C for infinity to hold the sample

Components of reaction mix of RAPD assay includes

a) Plant genomic DNA - 2.0 µl
b) 10 x Taq buffer A - 2.0 µl
c) dNTP mix (10 mM each) - 1.0 µl
d) $MgCl_2$ (25 mM) - 1.0 µl
e) Decamer primer (10 PM) - 1.5 µl

f) Taq DNA polymerase (IU) - 0.3 µl
g) Autoclaved distilled water - 12.2 µl
Total volume - 20.0 µl

RAPD assay was performed in a total reaction volume of 20 µl. A master mix was prepared for the required number of reactions by adding all the above components and given a spin for proper mixing. 2 µl of DNA was added individually into 200 µl PCR tube separately. Then 18 µl of master mix was pipetted out into these tubes. A brief spinning was given for the mix and then the tubes were loaded in the thermal cycles and the RAPD programme was run.

The amplified products were run on 1.2 per cent agarose gel and the gel was documented using gel documentation system.

3.2.2.5.2 Bulked Segregant Analysis (BSA)

Bulked segregant Analysis consists of pooling individuals from a segregating population according to two phenotypic classes DNAs from individuals belonging to same phenotypic character are pooled or mixed. The two resulting DNA bulks are equivalent to near isogenic lines and goal is to obtain molecular markers (RAPD) distinguishing these two DNA mixes. Based on the field reaction of F_2 population from both the crosses, the DNA isolated from 20 susceptible plants were pooled. 3 µl DNA from 20 susceptible F_2 plants of a cross were taken and pooled to make susceptible bulk/susceptible pool. The DNA isolated from twenty F_2 plants showing bacterial wilt resistance were also pooled separately and made resistant pool.

3.2.2.5.3 Selection of random primers for RAPD

Primers for detecting polymorphism were selected based on the RAPD assay conducted earlier in the department as a part of Sol Genome project. 18

primers were selected from OPA,OPF,OPAZ,OPY,OPS,OPAH,OPBG,OPN series based on earlier RAPD assay and primers were used to identify the primer giving polymorphism between parents, F_1, F_2susceptibe bulk and F_2 resistant bulk using thermal cycler.The primer giving specific polymorphism between resistant and susceptible parent was selected. Table 2. shows the nucleotide sequence of each of the 18 primers assayed.

After detection of the primer showing polymorphism that particular primer was utilized for further characterization of F_2 population.

3.2.2.5.4 Analysis of RAPD amplification profiles

Amplification profiles of parents, F_1 and F_2 population were compared with each other and the bands of DNA were scored manually as (1) or (0) depending on the presence or absence of particular band respectively and the segregation pattern of specific band related to bacterial wilt resistance in F_2 population was analyzed.

Table 2. Nucleotide sequences of primers selected for RAPD assay

Sl. No.	Primer	Nucleotide Sequence (5' – 3')
1	OPAZ 4	GTTTATCTCC
2	OPAZ 9	TGATCCCTGG
3	OPAZ16	CATCATCCTG
4	OPAZ17	GGACTGGAGT
5	OPAZ18	TCGGCCCTTC
6	OPF 3	CCTGATCACC
7	OPF 6	GGTGACGCAG
8	OPF 9	CCAAGCTTCC
9	OPF 16	GGAGTACTGG
10	OPAH12	CTGCTGGGAC
11	OPY 7	AGAGCCGTCA
12	OPY 8	AGGCAGAGCA
13	OPY 10	CAAACGTGGG
14	OPS 1	GTTTCGCTCC
15	OPS 3	CATCCCCCTG
16	OPS 8	GTCCACACGG
17	OPS 12	CCTTGACGCA
18	OPS 16	TTTGCCCGGA

Results

4. RESULTS

The results of the study conducted on molecular markers for bacterial wilt resistance in mapping populations of tomato at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Olericulture, College of Horticulture, Vellanikkara are presented in this chapter.

4.1 Development of segregating generation

Identified bacterial wilt resistant variety Anagha was crossed with two susceptible genotypes DVRT-1 and Pusa Ruby (Plate 2). F_1 plants of both the crosses along with the susceptible parents Pusa Ruby and DVRT-1 were raised in the bacterial wilt sick plot. All the F_1 plants and the susceptible parents Pusa Ruby and DVRT-1 succumbed to bacterial wilt within thirty days of transplanting. Anagha was resistant to bacterial wilt with a survival percentage of 92%.

Table 3.Evaluation of F_1 plants and their parents for resistance to bacterial wilt

Genotype	Survival percentage (Days after transplanting)				Disease reaction
	15	30	45	60	
Anagha	94.67	92	92	92	Resistant
DVRT-1	0	0	0	0	Susceptible
Pusa Ruby	0	0	0	0	Susceptible
AnaghaxDVRT-1 F_1	0	0	0	0	Susceptible
Anaghax Pusa Ruby F_1	0	0	0	0	Susceptible

Another set of F_1 plants were raised in pots filled with sterilized medium as all the F_1 plants could be susceptible to bacterial wilt (Plate 3). These F_1 plants were selfed to produce F_2 population of both the crosses. The fruits were harvested at red ripe stage and seeds were extracted (Plate 4).

Plate 2. Tomato parent genotypes raised in pots

a. F_1 genotypes at seedling stage

b. F_1 genotypes transplanted to individual pots in green house

Plate 3.Growth stages of F_1 genotypes (Anagha X DVRT-1 and

Anagha X Pusa Ruby)

Plate 4. F_1 plants of the crosses Anagha x Pusa Ruby and Anagha x DVRT-1 after fruit set

4.2 Evaluation of F_2 population for bacterial wilt resistance in the field

F_2 populations of the two crosses were screened for bacterial wilt in a wilt sick field. Bacterial wilt incidence was confirmed by ooze test. The symptom started as leaf drooping followed by complete wilting and death of the plant (Plate 5a). About 90 percentage of the wilted plants showed positive response to bacterial ooze test. When sliced stem of the wilted plant was placed in a test tube containing clear water and allowed to stand for few minutes, streaming of bacteria was observed confirming the presence of bacteria (Plate5b). Wilt incidence was recorded in the F_2 population everyday and the incidence at the frequency of 15 day interval is presented in Table 4.

Table 4. Bacterial wilt incidence in F_2 population

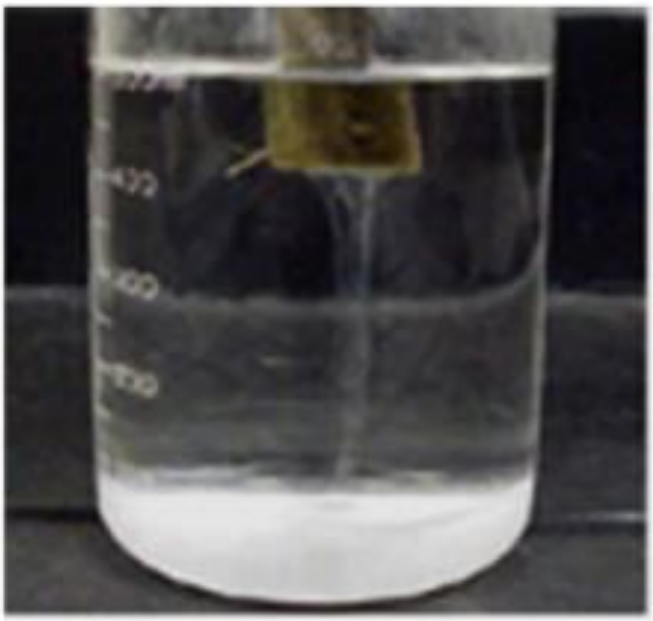
Cross	Per cent wilt incidence(Days after transplanting)				
	15	30	45	60	75
Anagha x Pusa Ruby	16	43	65	67.7	67.7
Anagha x DVRT-1	23	52	78	84.9	84.9

Wilting started one week after transplanting. Maximum wilt incidence was observed between thirty to forty five days after transplanting. There was no wilting 60 days after transplanting.

Among the two crosses, wilt incidence was more for the F_2 population of Anagha x DVRT-1(Table 4, 5 and Plate 6). Out of 200 seedlings transplanted, 172 plants showed wilt symptoms of which 157 showed positive reaction and 15 were negative to ooze test. These 15 plants exhibited wilt symptoms, but showed negative reaction to ooze test. Twenty eight plants did not show any wilt symptoms and these plants were resistant to bacterial wilt in the field.

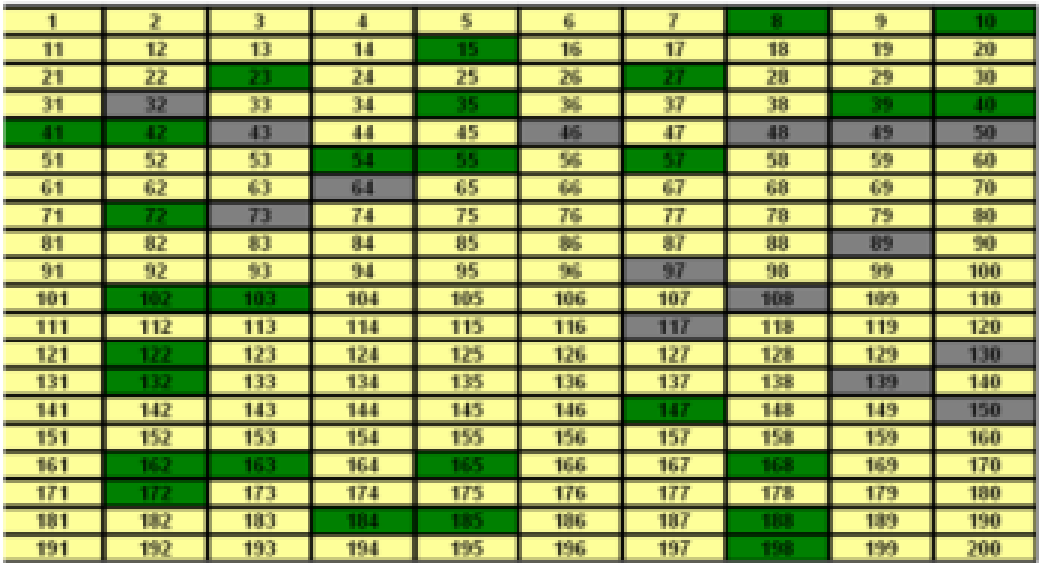
Wilting was less for the F_2 population of Anagha x Pusa Ruby. Out of 200 seedlings transplanted, 138 plants showed wilt symptoms of which 130 showed

a. Complete wilting of the tomato plant



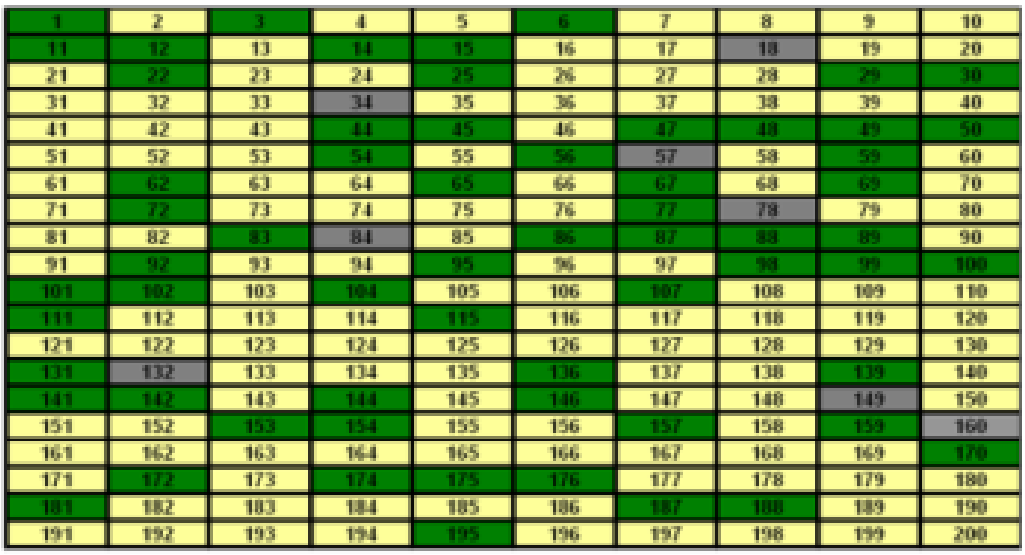
b.Bacterial ooze from wilt affected plant

Plate 5.Symptoms of bacterial wilt and its confirmation



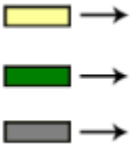
1	2	3	4	5	6	7	8	9	10
11	12	13	14	15	16	17	18	19	20
21	22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39	40
41	42	43	44	45	46	47	48	49	50
51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70
71	72	73	74	75	76	77	78	79	80
81	82	83	84	85	86	87	88	89	90
91	92	93	94	95	96	97	98	99	100
101	102	103	104	105	106	107	108	109	110
111	112	113	114	115	116	117	118	119	120
121	122	123	124	125	126	127	128	129	130
131	132	133	134	135	136	137	138	139	140
141	142	143	144	145	146	147	148	149	150
151	152	153	154	155	156	157	158	159	160
161	162	163	164	165	166	167	168	169	170
171	172	173	174	175	176	177	178	179	180
181	182	183	184	185	186	187	188	189	190
191	192	193	194	195	196	197	198	199	200

a. Reaction of F_2 population of Anagha x DVRT-1



1	2	3	4	5	6	7	8	9	10
11	12	13	14	15	16	17	18	19	20
21	22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39	40
41	42	43	44	45	46	47	48	49	50
51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70
71	72	73	74	75	76	77	78	79	80
81	82	83	84	85	86	87	88	89	90
91	92	93	94	95	96	97	98	99	100
101	102	103	104	105	106	107	108	109	110
111	112	113	114	115	116	117	118	119	120
121	122	123	124	125	126	127	128	129	130
131	132	133	134	135	136	137	138	139	140
141	142	143	144	145	146	147	148	149	150
151	152	153	154	155	156	157	158	159	160
161	162	163	164	165	166	167	168	169	170
171	172	173	174	175	176	177	178	179	180
181	182	183	184	185	186	187	188	189	190
191	192	193	194	195	196	197	198	199	200

b. Reaction of F_2 population of Anagha x Pusa Ruby



Susceptible genotypes (Positive reaction to ooze test)

Resistant genotypes

Plants wilted due to reasons other than bacterial wilt (Negative to ooze test)

Plate 6. Field reaction of F_2 population of Anagha x DVRT-1 and Anagha x Pusa Ruby to bacterial wilt

positive reaction and eight were negative to ooze test. These eight plants exhibited the symptom of wilt disease, but showed negative reaction to ooze test. 62 plants were resistant and did not show any wilt symptoms (Plate 7).

Table 5. Reaction of F_2 population to bacterial wilt in the bacterial wilt sick plot

Cross	Total number of plants	Susceptible genotypes(positive reaction to ooze test)	Wilted due to reasons other than bacterial wilt (negative reaction to ooze test)	Resistant genotypes
Anagha x DVRT-1	200	157	15	28
Anagha x Pusa Ruby	200	130	8	62

4.3 MOLECULAR CHARACTERIZATION

Genomic DNA of parents, F_1 and F_2 populations was isolated, purified and subjected to RAPD assay for molecular characterization.

4.3.1 Isolation and quantification of genomic DNA

DNA from tender tomato leaves were isolated using DNA isolation kit from Chromos Biotech Pvt. Ltd and Rogers and Bendich (1994) protocol with some modifications. The quality of DNA isolated by both the methods was tested using agarose gel electrophoresis. Better quality DNA with very distinct bands was obtained in DNA isolated using plant DNA isolation kit because RNA and protein contamination were totally absent. In the DNA samples isolated by Rogers and Bendich protocol, RNA contamination was observed.

a. F_2 population of the crosses Anagha X DVRT-1 and Anagha X Pusa Ruby in the field

b. Resistant F_2 plant after fruit set

Plate 7. F_2 population grown in the field

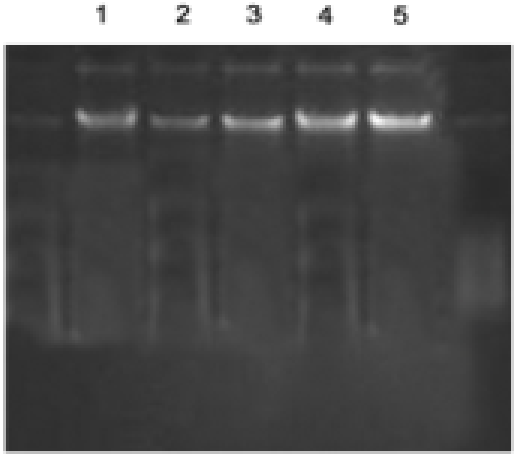
RNAse treatment was given to those DNAs isolated using Rogers and Bendich protocol in order to remove RNA (Sambrook *et al.*, 1989). After RNAse treatment uniform discrete bands were obtained for the samples indicating good quality DNA free from RNA and other contaminants (Plate 8 and Plate 9).

During isolation DNA from 28 plant samples of F_2 population of Anagha x DVRT-1and 32 DNA samples of Anagha x Pusa Ruby were degraded. Degraded DNA and DNA from plants wilted for reasons other than bacterial wilt were discarded. For further studies DNA from 157 F_2 population of Anagha x DVRT-1 and 160 F_2 population of Anagha x Pusa Ruby were used.

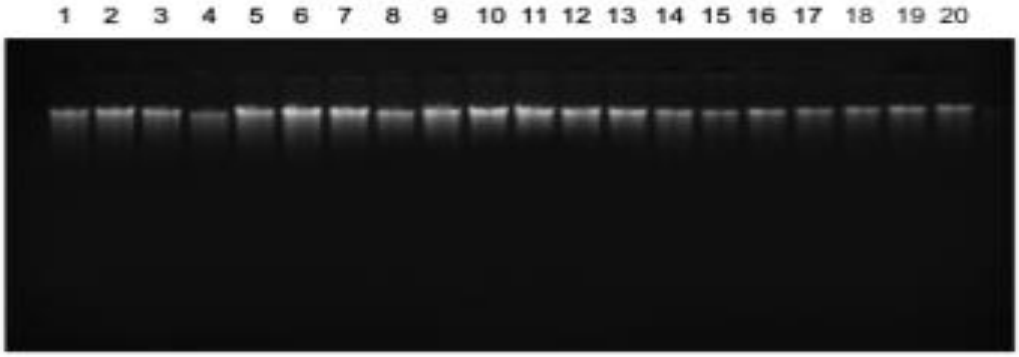
Table 6. Observation of F_2 population after discarding degraded DNA and DNA of F_2 plants that showed wilt symptoms but negative in reaction to ooze test

Cross	Total number of plants	Susceptible genotypes	Resistant genotypes
Anagha x DVRT- 1	157	130	27
Anagha x Pusa Ruby	160	110	50

After discarding the degraded DNA and DNA of F_2 plants that showed wilt symptoms but negative in reaction to ooze test, 157 DNA were obtained in the F2 population of the cross Anagha x DVRT- 1.Out of the 157 DNA, 130 DNA were from susceptible genotypes and 27 DNA were from resistant genotypes. In the F_2 population of the cross Anagha x Pusa Ruby 160 DNA were obtained after discarding degraded DNA and DNA of F_2 plants that showed wilt symptoms but negative in reaction to ooze test. Out of the 160 DNA, 110 DNA were from susceptible genotypes and 50 DNA were from resistant genotypes.



DNA isolated from Anagha (1), DVRT-1 (2), Pusa Ruby (3) Anagha X DVRT-1 F_1 (4), Anagha X Pusa Ruby F_1 (5)



DNA isolated from F_2 Population of the cross Anagha X DVRT-1

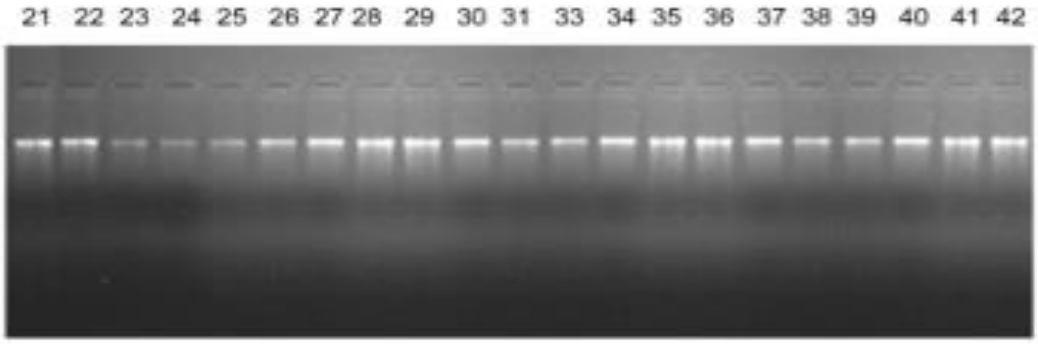


Plate 8. DNA isolated from F_2 Population of the cross Anagha X DVRT-1

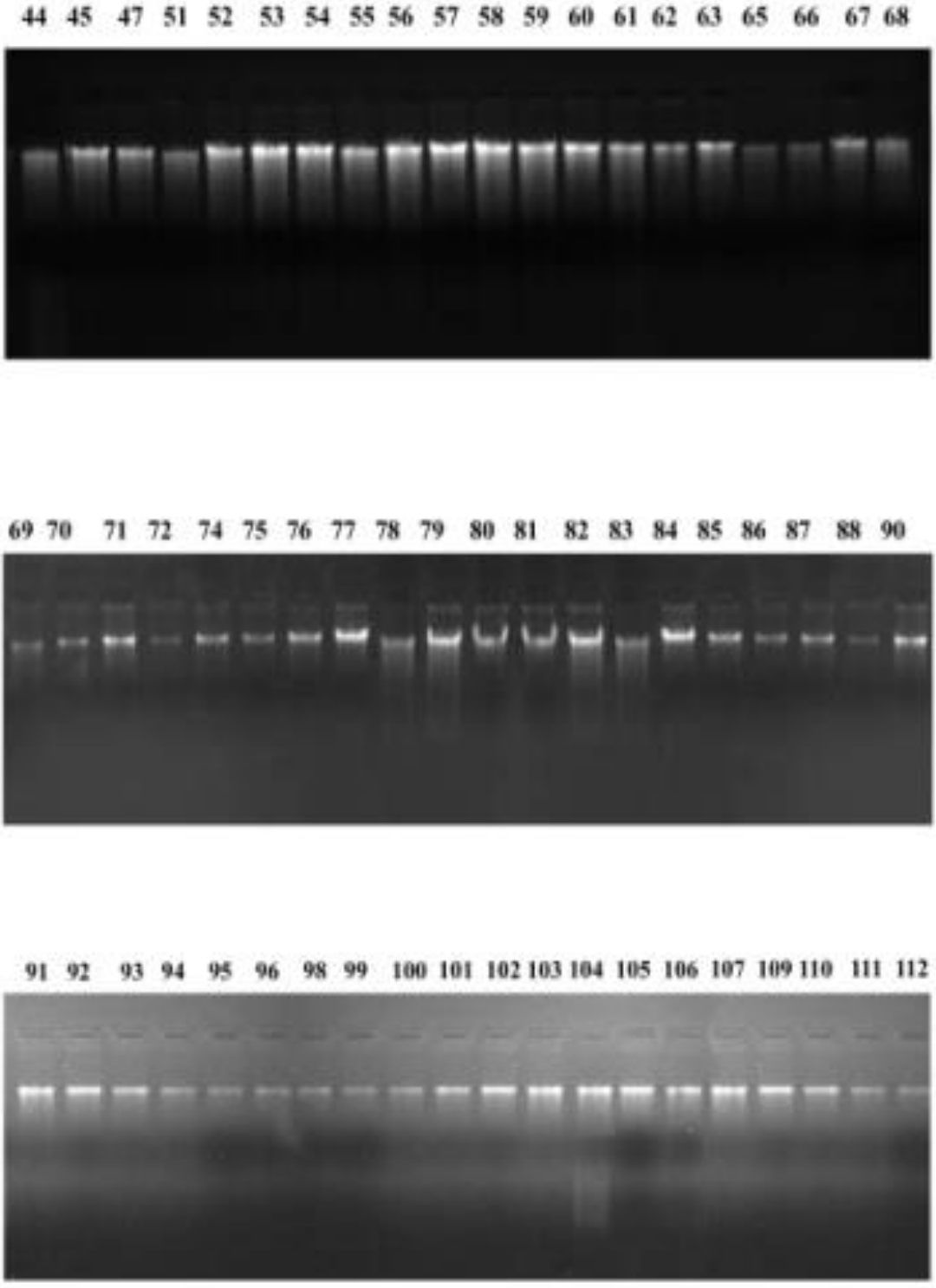


Plate 8. Continued

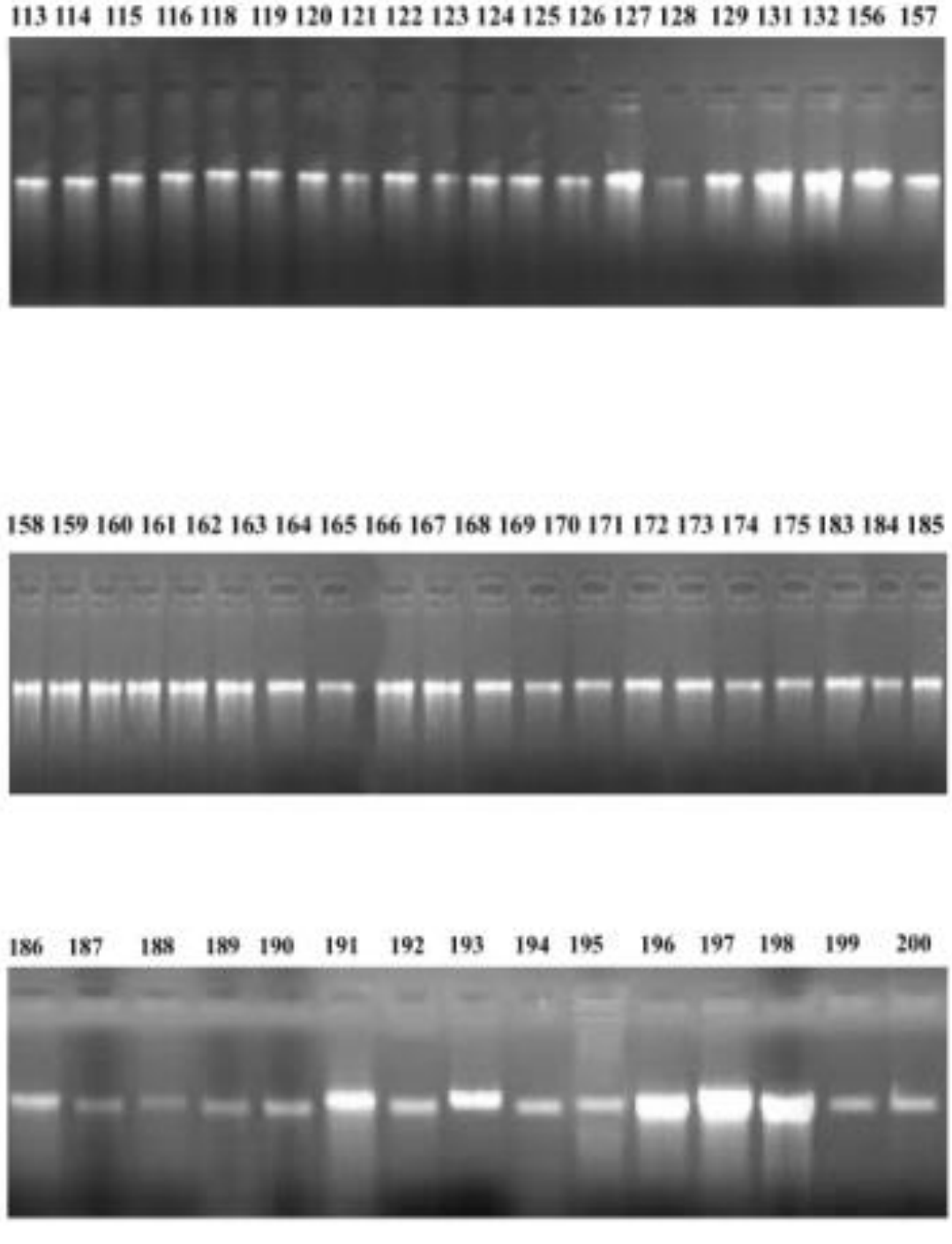


Plate 8. Continued

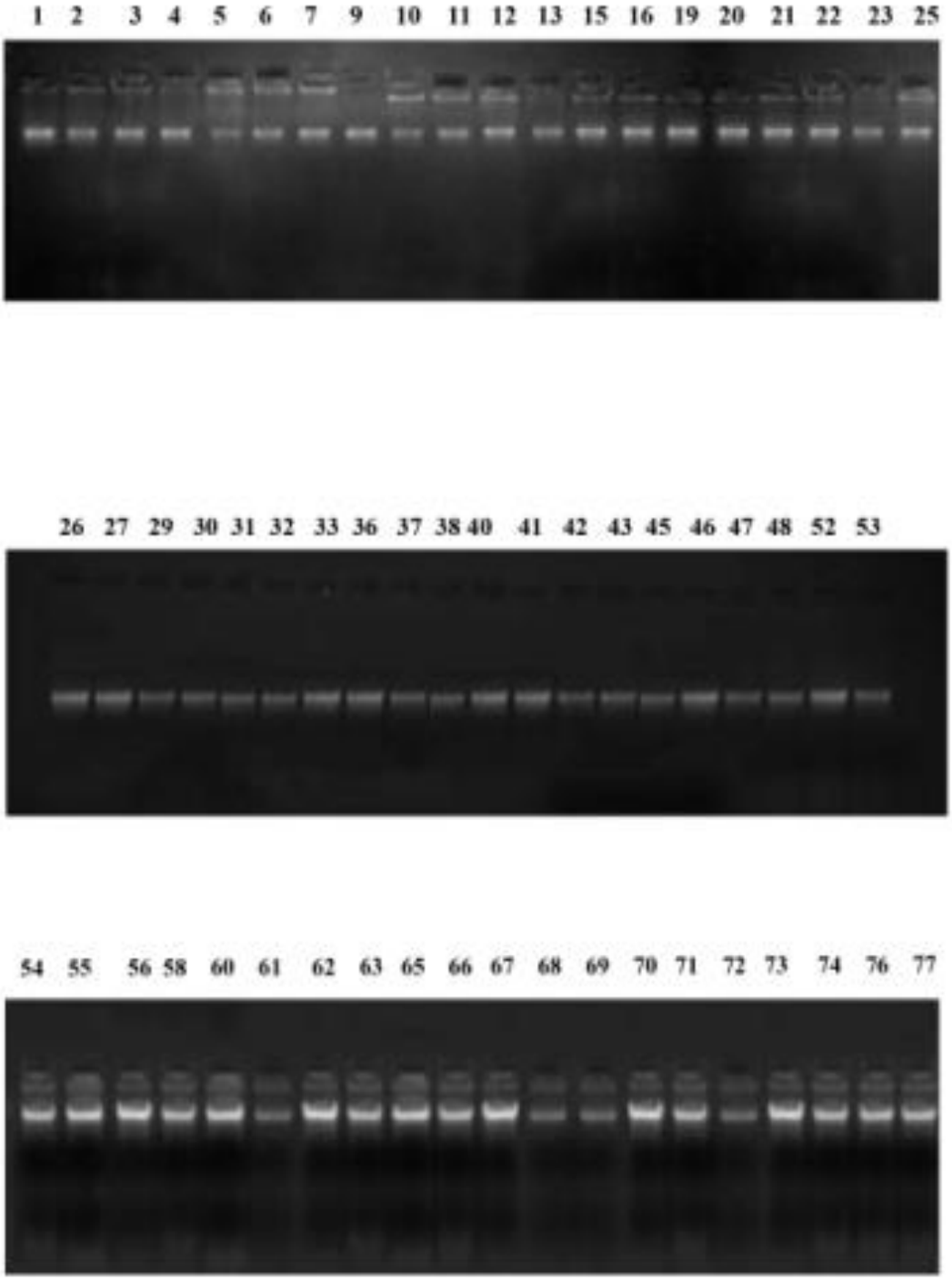


Plate 9. DNA isolated from F_2 Population of the cross Anagha x Pusa Ruby

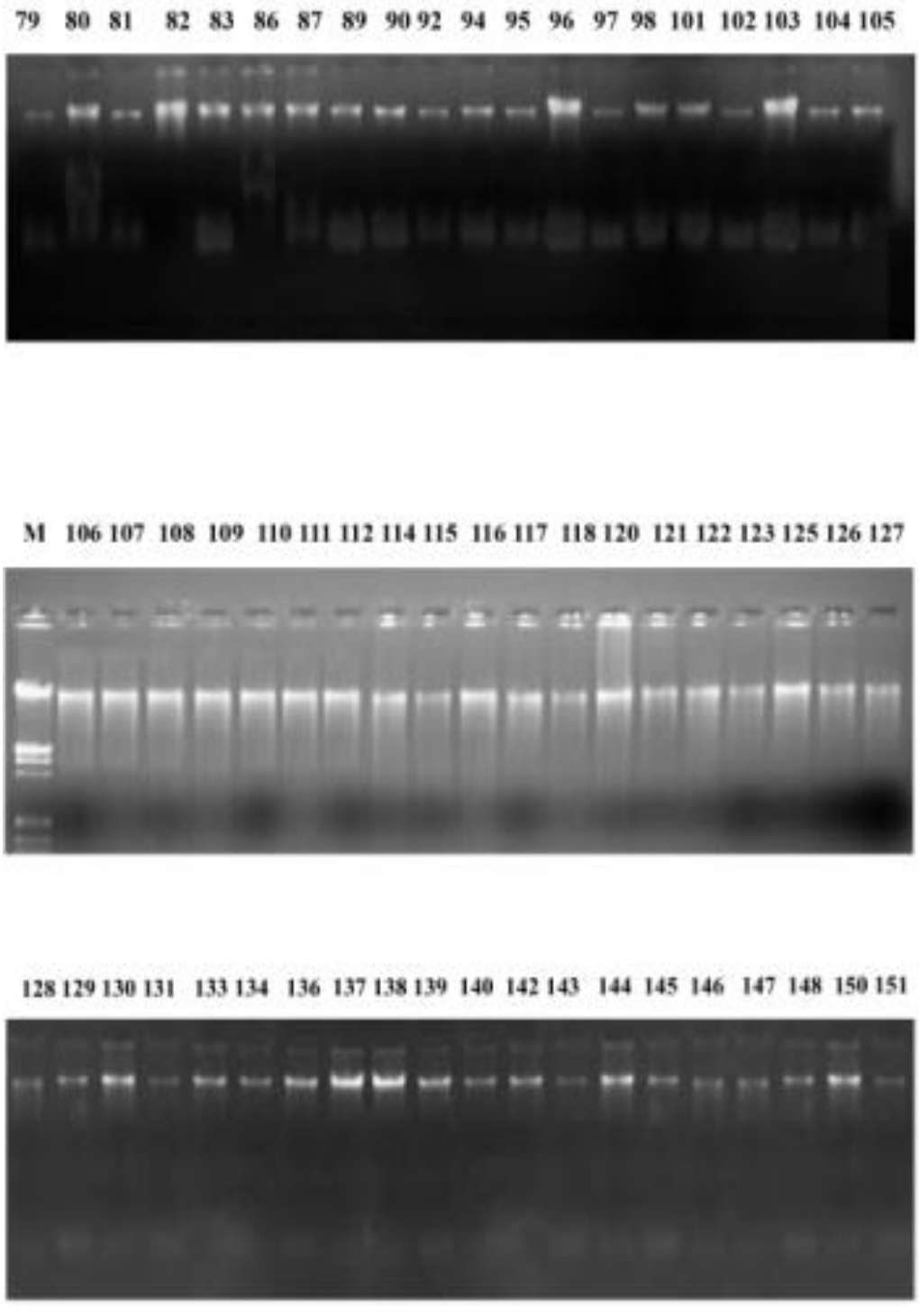


Plate 9. Continued

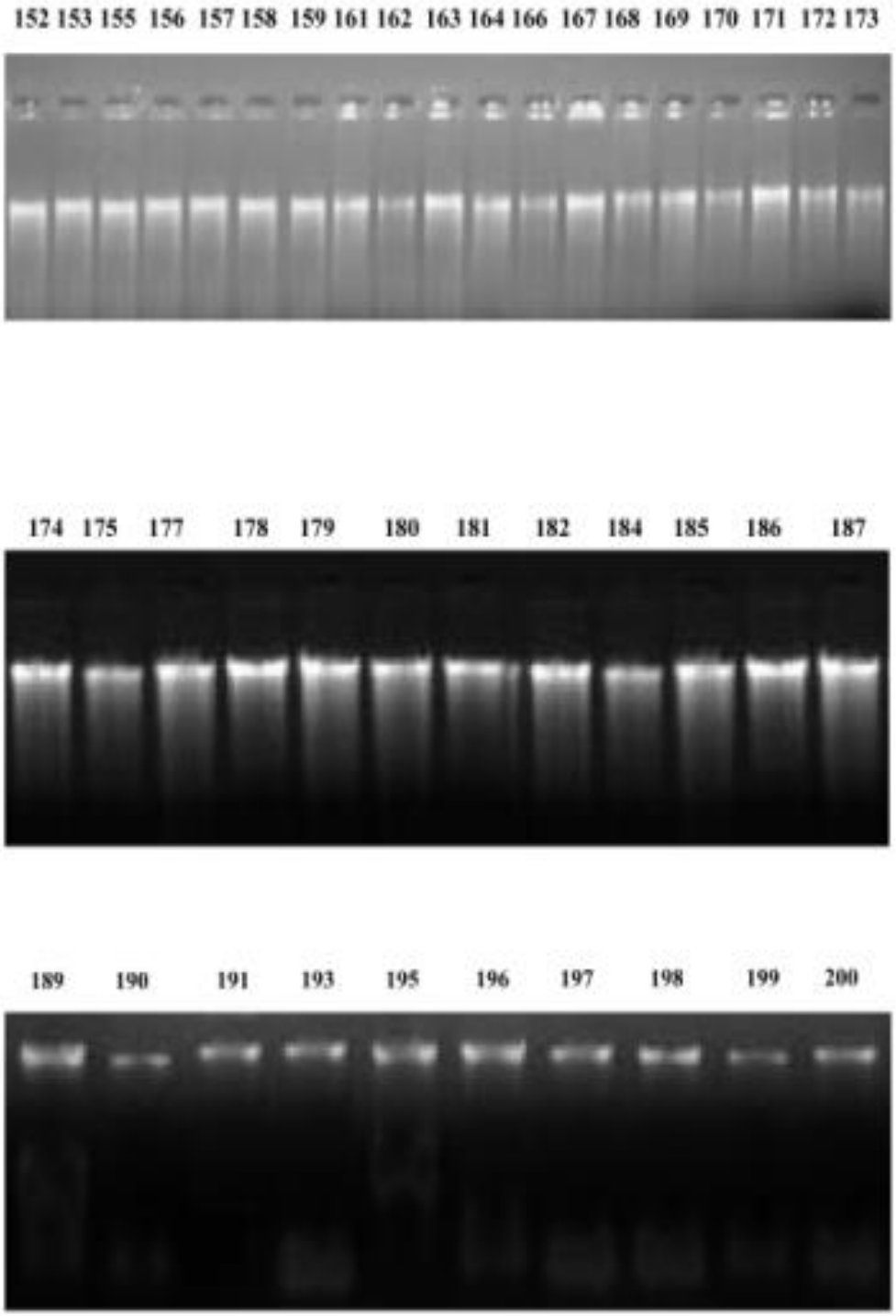


Plate 9. continued

The quality and quantity of DNA isolated using Rogers and Bendich method (1994) and plant DNA isolation kit was checked with Nanodrop Spectrophotometer. The ratio of absorbance at 260 nm and 280 nm ranged from 1.80 to 1.90 in DNA isolated by both the methods, indicating relatively pure DNA in the samples free from RNA and other contaminants like protein and pigments.The quantity of DNA ranged between 75 to 110 ng/ µl.The quality and quantity of DNA isolated from each genotype are given in the Table 7.

4.2.2 Random Amplified Polymorphic DNA (RAPD) assay

RAPD assay was carried out with good quality DNA isolated from parents, F_1, F_2 susceptible bulk and F_2 resistant bulk using selected RAPD primers.

4.2.2.1 RAPD profiles of tomato genotypes

Resistant parent, susceptible parent, F_1, F_2 susceptible bulk and F_2 resistant bulk of both the crosses, differing in reaction to bacterial wilt disease were characterized using 18 selected primers from 200 primers of OPF, OPAZ, OPS, OPAH, and OPY series.

In the case of Anagha x Pusa Ruby cross,DNA of Anagha, Pusa Ruby, Anagha x Pusa Ruby F_1, Anagha x Pusa Ruby F_2susceptibe bulk and Anagha x Pusa Ruby F_2 resistant bulk etc used for RAPD assay. In the case of Anagha x DVRT- 1 cross,DNA of Anagha, Pusa Ruby, Anagha x DVRT- 1 F_1, Anagha x DVRT- 1 F_2susceptibe bulk and Anagha x DVRT- 1 F_2 resistant bulk etc used for RAPD assay.

Table 7.Quantity and quality of DNA isolated from different genotypes

Genotype	Absorbance at 260 nm	Absorbance at 280 nm	Absorbance 260/280	Quantity (ng/µl)
Anagha	2.092	1.149	1.82	104.60
Pusa Ruby	2.101	1.160	1.81	105.05
DVRT-1	2.017	1.067	1.89	100.85
F_1 (Anagha x Pusa Ruby)	1.893	1.017	1.86	94.65
F_1 (Anagha xDVRT - 1)	1.907	1.047	1.82	95.35
F_2 Population of Anagha x DVRT-1				
1	2.093	1.102	1.90	104.63
2	1.812	0.966	1.88	90.58
3	1.775	0.954	1.86	88.75
4	1.608	0.874	1.84	80.40
5	2.088	1.130	1.84	104.41
6	2.071	1.101	1.88	103.55
7	1.799	0.950	1.89	89.95
8	1.770	0.950	1.86	88.50
9	1.652	0.904	1.83	82.60
10	1.775	0.943	1.88	88.75
11	1.662	0.909	1.83	83.12
12	2.089	1.098	1.90	104.45
13	1.764	0.943	1.87	88.22
14	1.650	0.899	1.84	82.51
15	2.071	1.101	1.84	103.55
16	2.088	1.130	1.81	104.40
17	1.763	0.946	1.86	88.15
18	1.790	0.952	1.88	89.52
19	1.812	0.966	1.83	90.60
20	1.652	0.904	1.81	82.60
21	1.770	0.939	1.88	88.50
22	1.799	0.950	1.89	89.95
23	1.775	0.943	1.86	88.75
24	1.812	0.966	1.88	90.58
25	1.875	0.994	1.88	93.75
26	1.987	1.098	1.81	99.35
27	2.018	1.118	1.81	100.91
28	1.927	1.025	1.88	96.35
29	1.853	1.001	1.85	92.65
30	1.839	1.01	1.82	91.95
31	1.875	1.024	1.83	93.75
33	1.732	0.921	1.88	86.62
34	1.589	0.879	1.80	79.45

35	1.602	0.880	1.82	80.11
36	1.783	0.924	1.92	89.15
37	1.508	0.799	1.88	75.42
38	1.654	0.901	1.84	82.70
39	14.32	7.657	1.87	716.1
40	1.578	0.850	1.86	78.90
41	1.569	0.861	1.82	78.45
42	16.83	9.146	1.84	841.5
44	1.711	0.914	1.87	85.55
45	1.628	0.884	1.84	81.43
47	1.709	0.894	1.91	85.45
51	1.579	0.846	1.86	78.95
52	1.593	0.874	1.82	79.65
53	1.692	0.924	1.83	84.66
54	1.619	0.879	1.84	80.95
55	1.643	1.125	1.85	82.15
56	2.013	1.108	1.85	100.65
57	1.723	0.941	1.83	86.15
58	1.637	0.880	1.86	81.85
59	1.573	0.841	1.87	78.65
60	1.771	0.952	1.86	88.55
61	1.854	0.991	1.87	92.71
62	1.836	0.992	1.85	91.86
63	1.978	1.06	1.86	98.93
65	2.092	1.149	1.82	104.67
66	2.112	1.135	1.86	105.64
67	1.909	1.01	1.89	95.45
68	2.145	1.178	1.82	107.25
69	2.173	1.187	1.83	108.65
70	2.157	1.198	1.80	107.85
71	2.099	1.134	1.85	104.95
72	1.973	1.090	1.81	98.65
74	2.172	1.174	1.85	108.6
75	1.956	1.045	1.87	97.85
76	2.133	1.165	1.83	106.65
77	2.083	1.138	1.83	104.15
78	1.862	1.001	1.86	93.13
79	1.939	1.053	1.84	96.95
80	1.681	0.918	1.83	84.05
81	1.796	0.976	1.84	89.82
82	2.124	1.141	1.86	106.2
83	1.937	1.070	1.81	96.85
84	1.916	1.024	1.87	95.80
85	20.54	11.10	1.85	1027.1

86	13.82	7.551	1.83	691.7
87	18.09	9.831	1.84	904.5
88	12.39	6.807	1.82	619.5
90	11.25	6.08	1.85	562.5
91	15.57	8.508	1.83	778.5
92	16.80	9.146	1.84	841.5
93	9.528	5.122	1.86	474.4
94	14.30	7.657	1.87	716.2
95	19.23	10.56	1.82	961.5
96	16.89	9.080	1.86	844.5
98	12.52	6.841	1.83	626.9
99	10.67	5.798	1.84	533.5
100	8.423	4.653	1.81	421.1
101	11.73	6.44	1.82	586.5
102	11.73	6.44	1.82	586.5
103	13.44	7.264	1.85	672.3
104	14.64	8.08	1.81	732.5
105	13.83	7.551	1.83	691.4
106	1.572	0.839	1.87	78.61
107	1.654	0.912	1.81	82.72
109	1.538	0.852	1.80	76.94
110	1.638	0.903	1.81	81.91
111	1.569	0.861	1.82	78.45
112	1.810	0.983	1.84	90.50
113	1.972	1.077	1.83	98.60
114	1.957	1.046	1.87	97.85
115	1.634	0.878	1.86	81.73
116	1.874	1.029	1.82	93.75
118	2.022	1.104	1.83	101.1
119	1.912	1.044	1.83	95.62
120	1.890	1.027	1.84	94.53
121	2.127	1.149	1.85	106.35
122	2.039	1.090	1.87	101.95
123	2.118	1.163	1.82	105.9
124	1.985	1.061	1.87	99.25
125	1.944	1.074	1.81	97.26
126	1.926	1.035	1.86	96.38
127	2.101	1.160	1.81	105.05
128	1.877	1.025	1.83	93.85
129	1.893	1.017	1.86	94.65
131	1.928	1.053	1.83	96.44
132	1.696	0.921	1.84	84.8
156	1.990	1.052	1.89	99.51
157	1.868	0.998	1.87	93.49

158	1.638	0.91	1.80	81.95
159	1.910	1.026	1.86	95.52
160	1.721	0.927	1.85	86.05
161	1.642	0.912	1.80	82.17
162	1.829	1.01	1.81	91.45
163	1.683	0.935	1.80	84.15
164	1.875	1.002	1.87	93.75
165	1.933	1.028	1.88	96.65
166	1.762	0.937	1.88	88.13
167	1.879	1.021	1.84	93.95
168	1.943	1.05	1.90	89.75
170	1.958	1.06	1.84	97.90
171	1.852	0.995	1.86	92.62
172	1.838	0.967	1.90	91.93
173	1.874	0.986	1.90	93.72
174	2.037	1.125	1.81	101.85
175	2.106	1.15	1.83	105.3
183	2.117	1.15	1.84	105.85
184	1.966	1.08	1.82	98.36
185	1.853	1.01	1.82	92.65
186	1.936	1.05	1.84	96.82
187	1.999	1.08	1.85	99.95
188	1.829	1.01	1.81	91.45
189	1.819	0.97	1.87	90.95
190	1.988	1.068	1.86	99.43
191	1.873	1.06	1.86	93.65
192	1.799	0.98	1.83	89.95
193	1.737	0.959	1.81	86.85
194	1.869	1.02	1.82	93.45
195	1.882	1.01	1.87	94.18
196	1.917	1.025	1.87	95.85
197	1.942	1.06	1.83	97.19
198	1.939	1.06	1.82	96.95
199	1.872	1.022	1.83	93.62
200	1.834	1.00	1.83	91.71

F_2 Population of Anagha x Pusa Ruby

1	2.170	1.185	1.83	108.5
2	2.137	1.135	1.88	106.85
3	1.953	1.073	1.82	97.65
4	1.936	1.069	1.81	96.83
5	1.793	0.95	1.88	89.65
6	1.864	1.029	1.81	93.28
7	1.684	0.935	1.84	84.27
9	1.971	1.059	1.86	98.55

10	2.093	1.150	1.82	104.65
11	1.919	1.06	1.81	95.75
12	1.853	1.012	1.83	92.65
13	1.777	0.976	1.82	88.85
15	2.017	1.067	1.89	100.85
16	1.632	0.86	1.88	81.63
19	1.771	0.95	1.86	88.55
20	1.669	0.92	1.80	83.45
21	1.653	0.913	1.81	82.65
22	1.947	1.03	1.88	97.35
23	1.972	1.077	1.83	78.64
25	1.622	0.886	1.83	81.19
26	2.101	1.160	1.81	105.05
27	1.912	1.044	1.83	95.65
29	2.022	1.104	1.83	101.1
30	1.696	0.921	1.84	84.83
31	1.633	0.878	1.86	81.77
32	1.892	1.027	1.84	94.54
33	14.64	8.08	1.81	732.3
36	1.879	1.026	1.83	92.95
37	1.946	1.057	1.84	97.34
38	1.972	1.048	1.88	98.66
40	2.162	1.14	1.89	108.10
41	13.44	7.264	1.85	672.5
42	1.699	0.94	1.80	85.95
43	1.773	0.979	1.81	88.65
45	1.937	1.035	1.87	96.35
46	2.029	1.09	1.86	101.45
47	1.788	0.98	1.82	89.45
48	1.913	1.034	1.85	96.65
52	1.857	1.01	1.83	92.85
53	1.992	1.070	1.86	99.64
54	11.73	6.44	1.82	586.5
55	2.106	1.108	1.90	105.3
56	2.099	1.53	1.82	104.9
58	1.872	1.038	1.82	93.64
60	1.893	1.095	1.81	94.65
61	1.695	0.926	1.83	84.75
62	1.698	0.917	1.85	84.94
63	1.573	0.839	1.87	78.61
65	1.962	1.055	1.86	98.15
66	1.810	0.983	1.84	90.58
67	1.876	1.025	1.83	93.86
68	1.912	1.025	1.86	95.62

69	1.808	0.966	1.87	90.42
70	11.73	6.44	1.82	586.5
71	1.869	1.02	1.82	93.45
72	1.998	1.08	1.85	99.96
73	2.105	1.15	1.83	105.4
74	1.936	1.05	1.84	96.82
76	1.791	0.98	1.83	89.95
77	1.834	1.00	1.83	91.76
79	1.942	1.06	1.83	97.17
80	1.883	1.01	1.87	94.13
81	1.820	0.97	1.87	90.95
82	1.939	1.06	1.82	96.95
83	1.885	1.813	1.86	94.25
86	1.929	1.059	1.82	96.45
87	1.993	1.065	1.87	99.65
89	2.072	1.12	1.85	103.6
90	2.117	1.138	1.86	105.8
92	1.762	0.973	1.81	88.12
94	1.847	1.020	1.81	92.35
95	1.852	1.012	1.83	92.69
96	1.973	1.090	1.81	98.65
97	10.67	5.798	1.84	533.5
98	1.763	0.974	1.81	88.15
101	1.642	0.907	1.81	82.14
102	1.854	0.991	1.87	92.72
103	1.635	0.893	1.83	81.75
104	1.759	0.956	1.84	87.95
105	1.771	.0937	1.89	88.55
106	2.156	1.198	1.80	107.8
107	1.662	0.893	1.86	83.12
108	1.946	1.040	1.87	97.34
109	1.833	0.992	1.85	91.85
110	1.672	0.918	1.82	83.67
111	1.539	0.840	1.83	76.95
112	1.612	0.890	1.81	80.67
114	1.579	0.848	1.86	78.95
115	1.796	0.981	1.83	89.82
116	1.856	1.019	1.82	92.81
117	1.839	1.01	1.82	91.95
118	2.027	1.083	1.87	101.35
120	1.739	0.960	1.81	86.95
121	8.423	4.653	1.81	421.1
122	1.927	1.030	1.87	96.35
123	2.046	1.10	1.86	102.3

125	1.907	1.047	1.82	95.35
126	1.578	0.850	1.86	78.92
127	1.879	1.026	1.83	92.95
128	1.909	1.048	1.82	95.45
129	1.914	1.057	1.81	95.70
130	1.692	0.924	1.83	84.68
131	1.732	0.921	1.88	86.66
133	1.578	0.850	1.86	78.91
134	1.783	0.850	1.86	78.95
136	1.508	0.799	1.88	75.43
137	1.593	0.84	1.82	79.65
138	19.23	10.56	1.82	961.5
139	1.812	0.966	1.88	90.58
140	1.980	1.05	1.87	99.00
142	2.075	1.109	1.87	103.75
143	2.018	1.118	1.81	100.91
144	1.668	0.916	1.82	83.42
145	1.875	0.994	1.88	93.75
146	1.952	1.055	1.85	97.65
147	1.637	0.909	1.80	81.85
148	9.528	5.122	1.86	474.4
150	1.749	0.966	1.81	87.45
151	9.528	5.122	1.86	474.4
152	1.825	0.991	1.84	91.25
153	2.091	1.136	1.84	104.5
155	1.933	1.022	1.89	96.65
156	16.80	9.146	1.84	841.5
157	1.877	1.009	1.86	93.85
158	1.799	0.977	1.84	89.95
159	1.698	0.917	1.85	84.92
161	1.808	0.966	1.87	90.47
162	1.912	1.025	1.86	95.63
163	15.57	8.508	1.83	778.5
164	1.885	1.030	1.83	94.25
166	1.907	1.047	1.82	95.35
167	1.794	0.969	1.85	89.75
168	1.876	1.025	1.83	93.81
169	1.963	1.055	1.86	98.15
170	1.683	0.909	1.85	84.15
171	1.994	1.101	1.81	99.74
172	1.634	0.883	1.85	81.72
173	1.806	0.947	1.90	90.38
174	1.856	1.014	1.83	92.81
175	1.934	1.062	1.82	96.73

177	11.25	6.08	1.85	562.5
178	2.072	1.12	1.85	103.6
179	1.646	0.889	1.85	82.36
180	1.711	0.914	1.87	85.55
181	2.071	1.101	1.88	103.5
182	1.897	1.036	1.83	94.85
184	1.958	1.058	1.85	97.93
185	1.739	0.767	1.87	86.95
186	12.39	6.807	1.82	619.5
187	1.944	1.074	1.81	97.22
189	2.063	1.12	1.83	103.1
190	1.683	0.909	1.85	84.15
191	1.833	0.980	1.87	91.65
193	18.09	9.831	1.84	904.5
195	1.806	0.947	1.90	90.31
196	1.783	0.850	1.86	78.93
197	1.508	0.799	1.88	75.42
198	1.579	0.848	1.86	78.95
199	1.799	0.977	1.84	89.95
200	1.885	1.030	1.83	94.25

4.2.2.1a) RAPD profile with selected primers from OPAZ primer series

Based on the RAPD assay done in Sol Genome project with Anagha, Pusa Ruby and DVRT-1, with different primers from OPBG, OPF, OPAZ, OPY, OPS, OPA, OPN series 18 primers were selected. The details of the amplification pattern generated by these primers are given below.

OPAZ 4

Six amplicons were obtained after DNA amplication with the primer OPAZ 4. Among six bands produced, five bands were clear and one band was faint. All the six bands produced were found to be monomorphic among the parents, F_1 and F_2 resistant and susceptible bulk of the two crosses (Plate 10a). The molecular weight of the bands obtained ranged between 1.54 kb and 564 bp.

OPAZ 9

RAPD assay with the primer OPAZ 9 displayed seven amplicons of which three were bright and four bands were faint. All the seven bands were monomorphic among the genotypes of the two crosses (Plate 10a) and this primer exhibited zero per cent polymorphism. The molecular size of the fragments ranged between 1.75 kb and .764 kb.

OPAZ 16

The RAPD profile generated by this primer displayed seven unique fragments of different sizes. The size of the amplicons ranged between 1.45 kb and .64kb. All the seven amplicons were present in Anagha, Pusa Ruby, DVRT-1, F_1plants and F_2 susceptible and resistant bulks of both the crosses (Plate 10a).

OPAZ 17

The primer OPAZ 17 gave only two bands when the amplification products were viewed on agarose gel electrophoresis after RAPD reaction (Plate 10a). There were one faint band and one clear bright band and they were of 1.47kb and .84kb respectively.

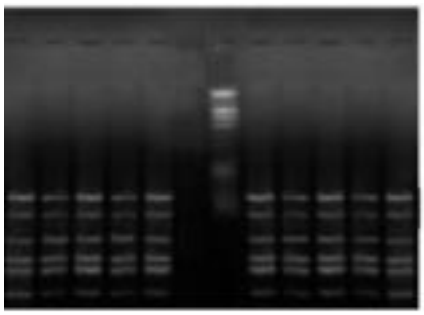
OPAZ 18

This primer produced four amplicons and the RAPD profile obtained with this primer is shown in Plate 10a. Unique bands shared by resistant/susceptible genotypes were not obtained and the size of the fragments ranged from 1.1kb to .2kb. Out of four amplicons one band was very faint and was not reproducible.

4.2.2.1b) RAPD profile with selected primers from OPF series

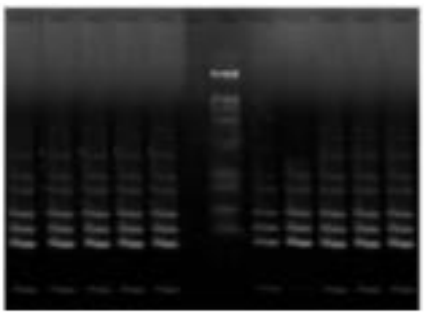
RAPD assay done in the Sol Genome Project with DNA isolated from Anagha, Pusa Ruby and DVRT-1 using OPF primer series were analyzed and selected four primers for detecting polymorphism.

1 2 3 4 5 B M 6 7 8 9 10



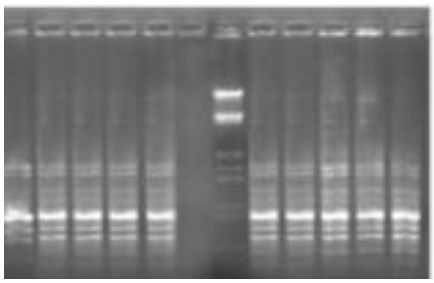
OPAZ 4

1 2 3 4 5 B M 6 7 8 9 10



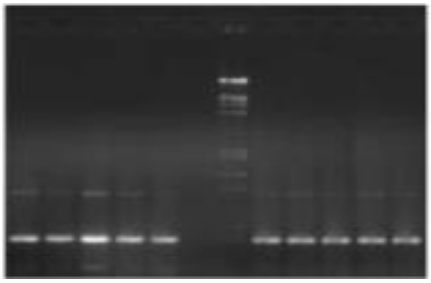
OPAZ 9

1 2 3 4 5 B M 6 7 8 9 10



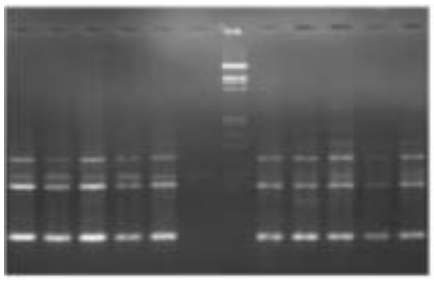
OPAZ 16

1 2 3 4 5 B M 6 7 8 9 10



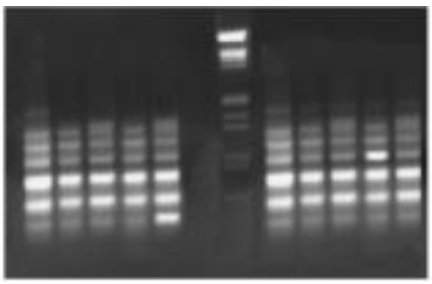
OPAZ 17

1 2 3 4 5 B M 6 7 8 9 10



OPAZ 18

1 2 3 4 5 B M 6 7 8 9 10



OPF 3

1. Anagha
2. Pusa Ruby
3. Anagha x Pusa Ruby F_1
4. Anagha x Pusa Ruby F_2 Susceptible bulk
5. Anagha x Pusa Ruby Resistant bulk
B- Blank

M - Marker
6. Anagha
7. DVRT-1
8. Anagha x DVRT-1 F_1
9. Anagha x DVRT-1 F_2 Susceptible bulk
10. Anagha x DVRT-1 F_2 Resistant bulk

Plate 10a. RAPD profile with primers OPAZ 4, 9, 16, 17, 18 and OPF 3

OPF 3

A total of seven amplicons were obtained after DNA amplification with the primer OPF 3. The pattern of amplification is shown in Plate 10a. One faint band was present in F_1 and resistant bulk of the two crosses, but there was no difference between resistant and susceptible parents. The molecular weight of the bands varied from 1.45 kb to .265 kb. Out of seven bands, two bands were very clear and bright, and the rest were faint.

OPF 6

Amplification with this primer generated six amplicons of which two were bright and four bands were faint. The molecular weight of the amplicons ranged between 2.1kb and .56kb. Unique bands shared by resistant / susceptible genotypes were not obtained (Plate10b).

OPF 9

Using OPF 9, five bands were obtained and were found to be monomorphic (Plate 10b). The size of the bands ranged from 1.55 kb to 467.8 bp. There were no unique bands that could differentiate the resistant and susceptible genotypes and all the five bands produced were clear and thick.

OPF 16

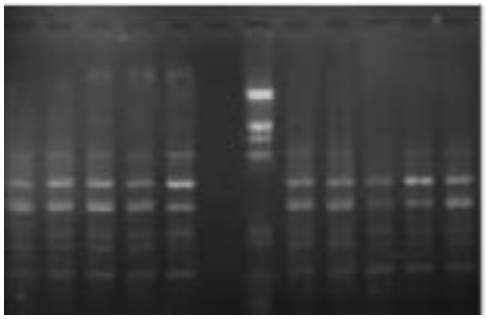
Nine amplicons were observed on the agarose gel for the DNA amplified with the primer OPF 16 (Plate 10b). This primer was also unable to differentiate between the resistant and susceptible genotypes. All the nine bands produced were monomorphic for both the crosses and fragment size ranged between 2.51 kb and 182 bp.

4.2.2.1c) RAPD profile with selected primer from OPAH series

OPAH 12

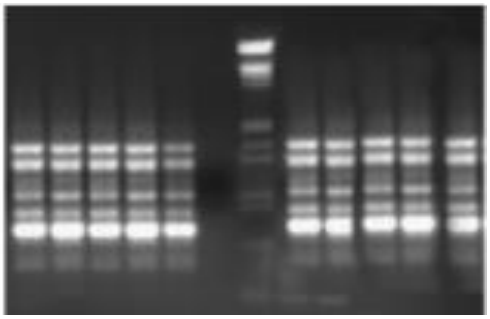
Nine amplicons were obtained with primer OPAH 12 on RAPD assay (Plate 10b). This primer was unable to distinguish between bacterial wilt resistant

1 2 3 4 5 B M 6 7 8 9 10



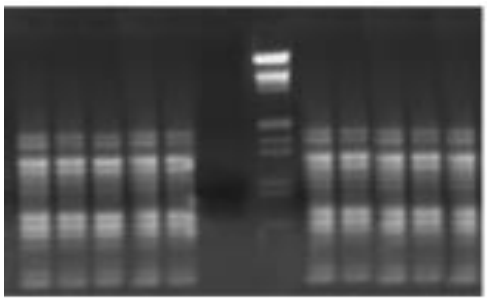
OPF 6

1 2 3 4 5 B M 6 7 8 9 10



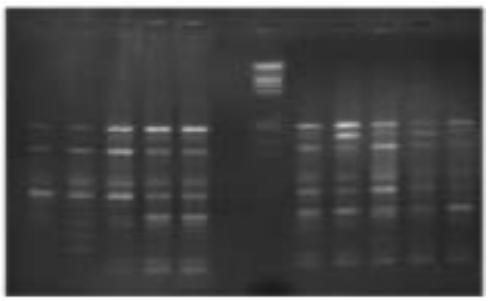
OPF 9

1 2 3 4 5 B M 6 7 8 9 10



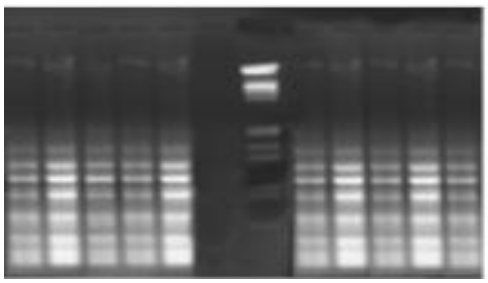
OPF 16

1 2 3 4 5 B M 6 7 8 9 10



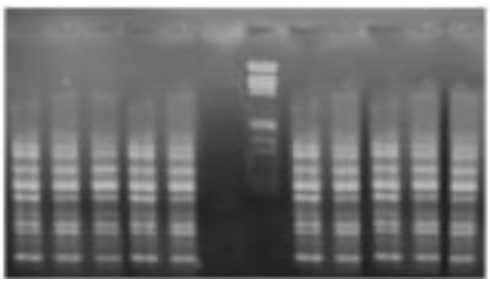
OPAH 12

1 2 3 4 5 B M 6 7 8 9 10



OPY 7

1 2 3 4 5 B M 6 7 8 9 10



OPY 8

1. Anagha

2. Pusa Ruby

3. Anagha x Pusa Ruby F_1

4. Anagha x Pusa Ruby F_2 Susceptible bulk

5. Anagha x Pusa Ruby Resistant bulk

B- Blank

M - Marker

6. Anagha

7. DVRT-1

8. Anagha x DVRT-1 F_1

9. Anagha x DVRT-1 F_2 Susceptible bulk

10. Anagha x DVRT-1 F_2 Resistant bulk

Plate 10b. RAPD profile with primers OPF 6, 9, 16 OPAH 12,OPY 7 and 8

nd susceptible genotypes. The fragments size ranged between 1.94kb and .26 kb. For the cross Anagha x DVRT-1 all the nine bands were present in all the genotypes. In the cross Anagha x Pusa Ruby four faint bands were common to F_2 resistant and susceptible bulk genotypes, but those bands were absent in Anagha and Pusa Ruby .

4.2.2.1d) RAPD profile with selected primers from OPY series

Based on the RAPD assays conducted in Sol Genome Project three primers from OPY series were selected and details on the amplification of the 3 primers OPY 7, 8, 10 are given below.

OPY 7

Nine amplicons were observed on the agarose gel for the DNA amplified with the primer OPY 7 (Plate 10b). Two bands were intense and bright, rest seven bands were not sharp. This primer also produces monomorphic bands in the two crosses and percentage polymorphism is zero. The molecular weight of the bands obtained ranged between 1.6 kb and .162bp.

OPY 8

Nine amplicons were obtained after DNA amplification with the primer OPY 8. All the bands produced were monomorphic for both the crosses and band size ranged from 1.57 kb to 109 bp (Plate 10b).

OPY 10

RAPD assay with this primer displayed three intense amplicons and four faint bands. RAPD profile generated by this primer is shown in Plate 10c. Unique bands shared by resistant/susceptible genotypes were not obtained. The fragments size ranged between 2.3 kb and 95 bp.

4.2.2.1e) RAPD profile with selected primers from OPS primer series

RAPD profile of the OPS primer series done in Sol Genome project was analyzed and five primers were selected from OPS primer kit to detect polymorphism. The amplification profile details with 5 selected OPS primers are given below.

OPS 1

All the three bands produced were found to be monomorphic among parents, F_1 and F_2 susceptible and resistant bulk of the two crosses i.e. Anagha x Pusa Ruby and Anagha x DVRT-1. The size of the amplicons ranged between 1.32 kb and .76kb and this primer exhibited zero polymorphism (Plate 10c).

OPS 3

The RAPD profile generated by this primer displayed six scorable fragments and four faint bands in all the genotypes. All the bands were monomorphic among the genotypes in both the crosses and the molecular size of the fragments ranged between 1.39 kb and 352 kb (Plate 10c).

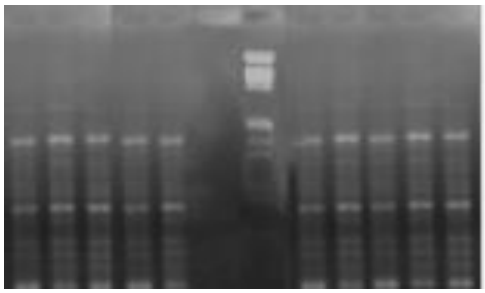
OPS 8

The primer gave a total of four intense and four faint bands. This primer was also unable to distinguish between bacterial wilt resistant and susceptible genotypes (Plate 10c). The size of the amplicons ranged between 1.8 kb and 263 bp and all the bands were found to be monomorphic.

OPS 12

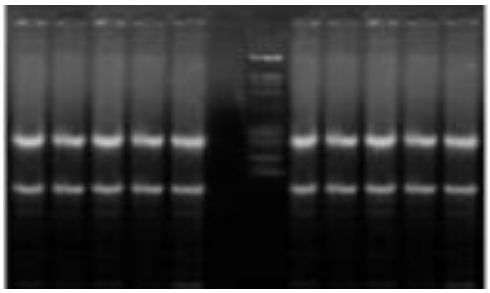
RAPD profile with the primer OPS 12 displayed eight amplification products and all the eight bands were present in all the genotypes. The size of the amplicons varied from 2.1 kb to 195 bp. The amplification pattern is shown in Plate 10c.

1 2 3 4 5 B M 6 7 8 9 10



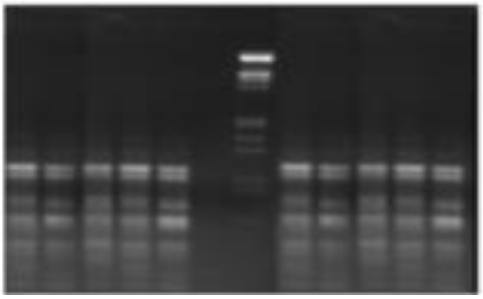
OPY 10

1 2 3 4 5 B M 6 7 8 9 10



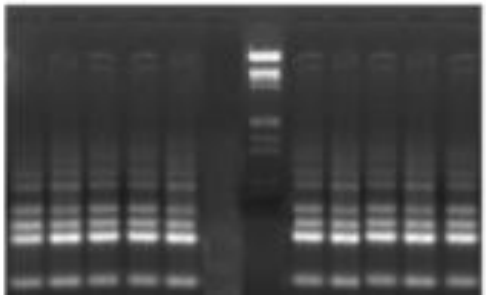
OPS 1

1 2 3 4 5 B M 6 7 8 9 10



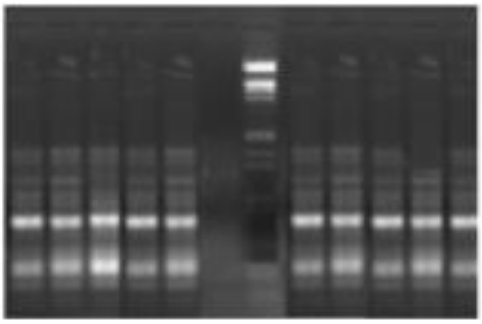
OPS 3

1 2 3 4 5 B M 6 7 8 9 10



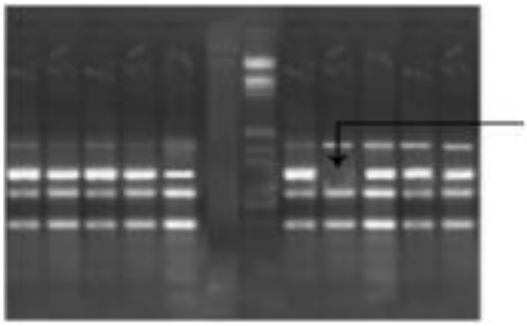
OPS 8

1 2 3 4 5 B M 6 7 8 9 10



OPS 12

1 2 3 4 5 B M 6 7 8 9 10



OPS 16

1. Anagha
2. Pusa Ruby
3. Anagha x Pusa Ruby F_1
4. Anagha x Pusa Ruby F_2 Susceptible bulk
5. Anagha x Pusa Ruby F_2Resistant bulk
B- Blank
M - Marker
6. Anagha
7. DVRT-1
8. Anagha x DVRT-1 F_1
9. Anagha x DVRT-1 F_2 Susceptible bulk
10. Anagha x DVRT-1 F_2 Resistant bulk

Plate 10c. RAPD profile with primers OPY 10, OPS 1,3,8,12,16

OPS 16

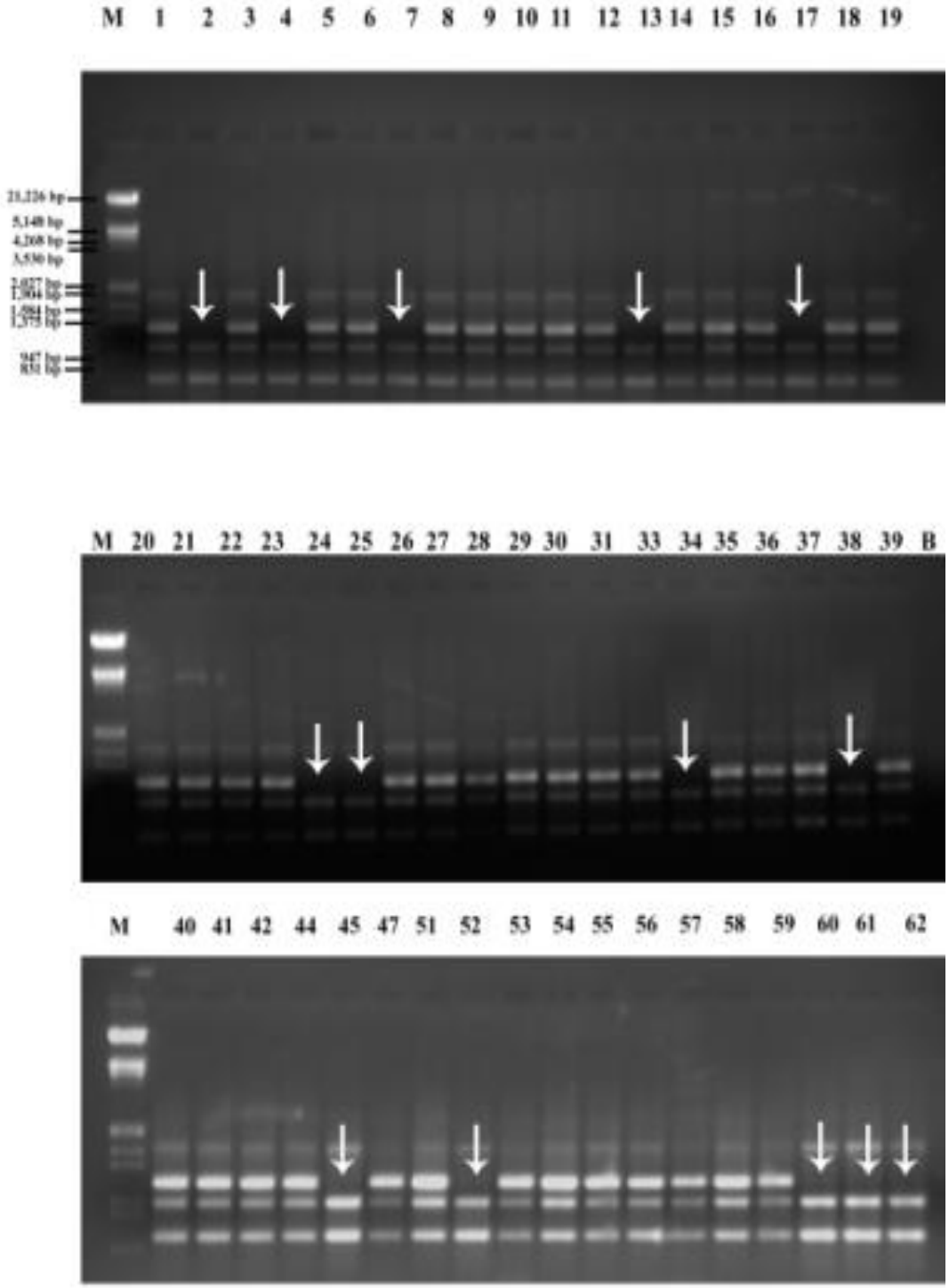
The primer OPS 16 generated four amplicons on RAPD analysis of the tomato genotypes of which one band is polymorphic between Anagha and DVRT-1. The bands ranged in molecular weight from 2.43 kb to 514 bp. A 1.36 kb band is present in Anagha, Anagha x DVRT-1 F_1, F_2 resistant bulk, F_2 susceptible bulk but absent in DVRT-1 (plate 10c).

Using the same primer OPS 16, polymorphism was not there between Anagha and Pusa Ruby. OPS 16 was not able to distinguish between Anagha and Pusa Ruby. All the four bands were present in Anagha, Pusa Ruby, F_1, F_2 susceptible bulk and F_2 resistant bulk of the cross Anagha X Pusa Ruby.So further study of segregation of F_2 population was carried out only in F_2 population of Anagha X DVRT -1 only.

4.2.2.2 Analysis of the mapping population with OPS 16

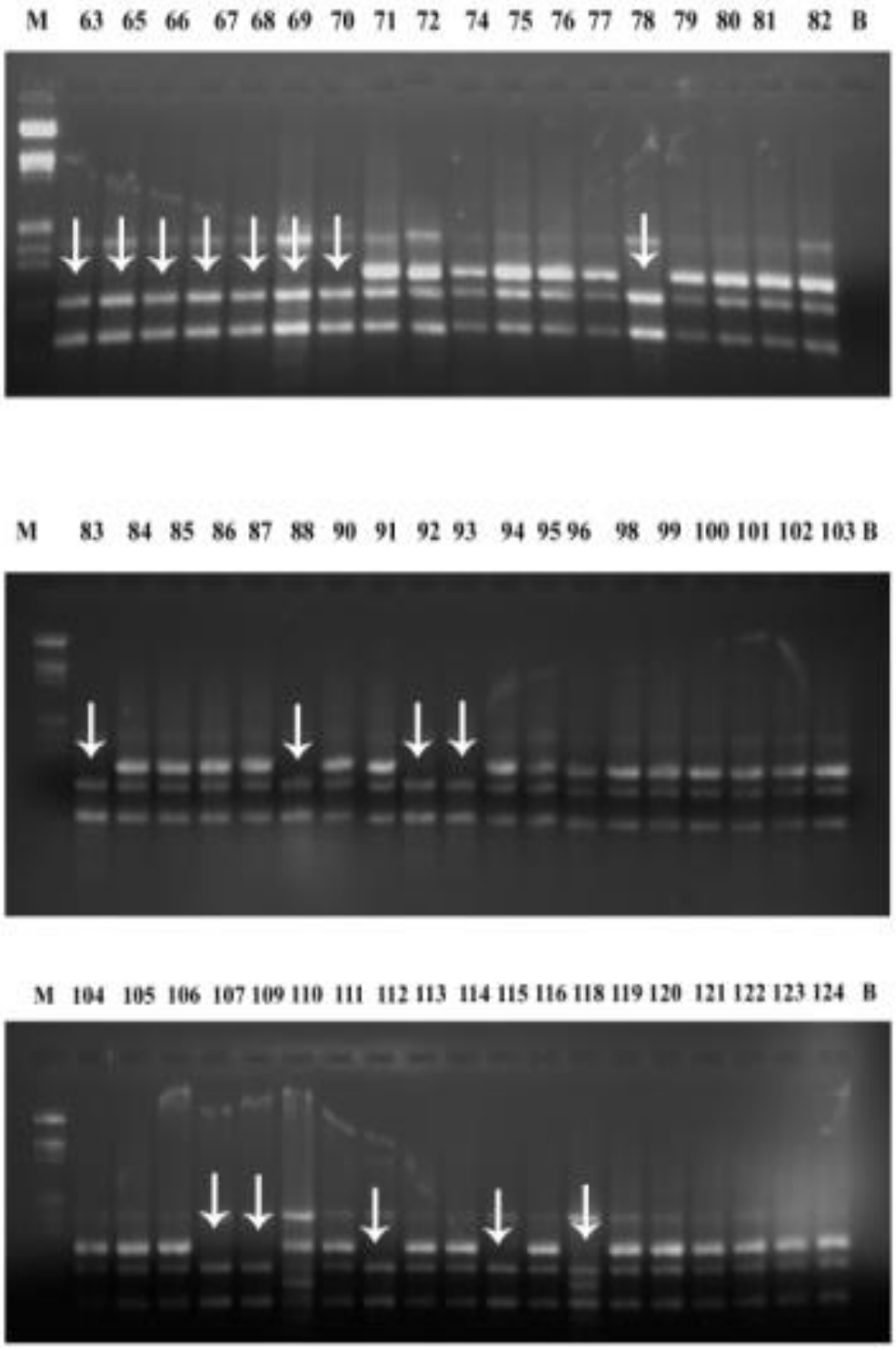
Whole F_2 population (157) of the cross Anagha x DVRT-1 was analyzed individually with the primer OPS 16, which gave polymorphism between Anagha and DVRT-1. Table 8 shows the scoring of the F_2 population of Anagha x DVRT-1 with respect to the polymorphic band obtained using OPS16.

Out of 157 plants, all the 27 plants which were found to be resistant in the field, gave 1.3 kb clear bright band in their RAPD profile during molecular characterization with OPS 16 primer. The rest 130 plants which were found to be susceptible in the field, showed two types of RAPD profiles. Out of 130 susceptible plants, 35 plants did not show 1.3 kb band in their amplification profile. Rest 95 plants gave 1.3 kb band while amplification pattern was observed on the agarose gel for the DNAs amplified with the primer OPS 16 (Plate 11). Amplification profile of the F_2 population of the cross Anagha x DVRT-1 were scored manually as (1) or (0) depending on the presence or absence of particular band respectively.



***Arrow indicates absence of polymorphic band**

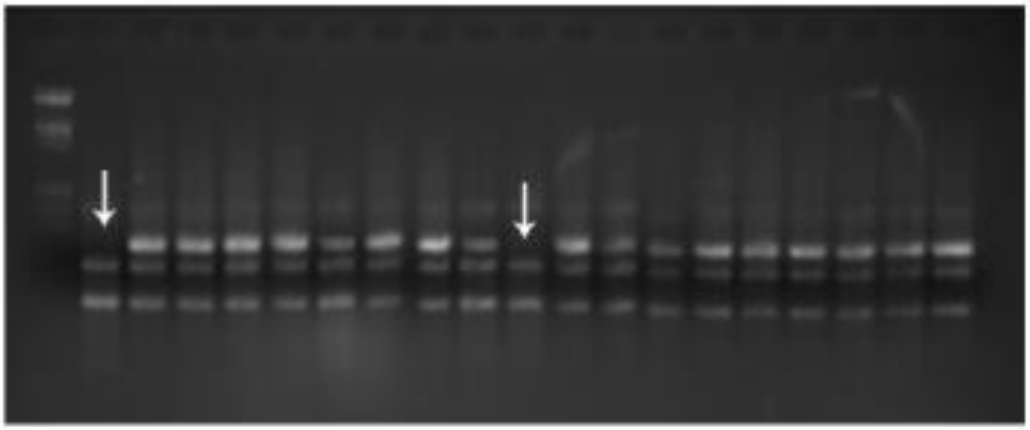
Plate 11.RAPD profile of the F2 Population of the cross Anagha x DVRT-1 with the primer OPS 16



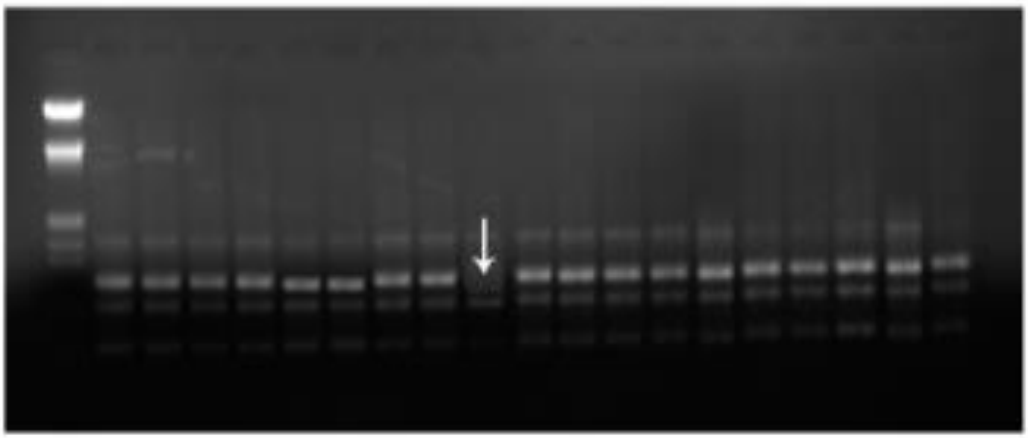
***Arrow indicates absence of polymorphic band**

Plate 11. continued

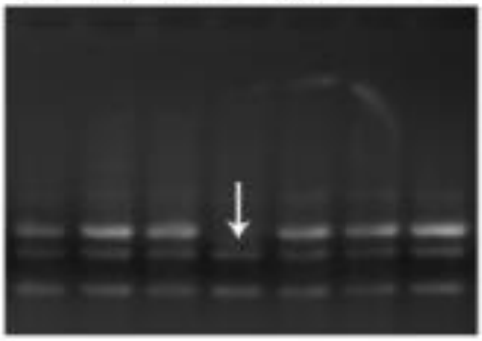
M 125 126 127 128 129 131 132 156 157 158 159 160 161 162 163 164 165 166 167



M 168 169 170 171 172 173 174 175 183 184 185 186 187 188 189 190 191 192 193



194 195 196 197 198 199 200



***Arrow indicates absence of polymorphic band**

Plate 11. continued

Table 8. Scoring of the F_2 population of Anagha x DVRT-1 with respect to the polymorphic band obtained using the primer OPS16

Genotype	Field reaction	Scoring based on RAPD assay using OPS16
1	S	1
2	S	0
3	S	1
4	S	0
5	S	1
6	S	1
7	S	0
8	R	1
9	S	1
10	R	1
11	S	1
12	S	1
13	S	0
14	S	1
15	R	1
16	S	1
17	S	0
18	S	1
19	S	1
20	S	1
21	S	1
22	S	1
23	R	1
24	S	0
25	S	0
26	S	1
27	R	1
28	S	1
29	S	1
30	S	1
31	S	1
33	S	1
34	S	0
35	R	1
36	S	1
37	S	1
38	S	0
39	R	1
40	R	1
41	R	1

42	R	1
44	S	1
45	S	0
47	S	1
51	S	1
52	S	0
53	S	1
54	R	1
55	R	1
56	S	1
57	R	1
58	S	1
59	S	1
60	S	0
61	S	0
62	S	0
63	S	0
65	S	0
66	S	0
67	S	0
68	S	0
69	S	0
70	S	0
71	S	1
72	R	1
74	S	1
75	S	1
76	S	1
77	S	1
78	S	0
79	S	1
80	S	1
81	S	1
82	S	1
83	S	0
84	S	1
85	S	1
86	S	1
87	S	1
88	S	0
90	S	1
91	S	1
92	S	0
93	S	0

94	S	1
95	S	1
96	S	1
98	S	1
99	S	1
100	S	1
101	S	1
102	R	1
103	R	1
104	S	1
105	S	1
106	S	1
107	S	0
109	S	0
110	S	1
111	S	1
112	S	0
113	S	1
114	S	1
115	S	0
116	S	1
118	S	0
119	S	1
120	S	1
121	S	1
122	R	1
123	S	1
124	S	1
125	S	0
126	S	1
127	S	1
128	S	1
129	S	1
131	S	1
132	R	1
156	S	1
157	S	1
158	S	0
159	S	1
160	S	1
161	S	1
162	R	1
163	R	1
164	S	1

165	R	1
166	S	1
167	S	1
168	R	1
170	S	1
171	S	1
172	R	1
173	S	1
174	S	1
175	S	1
183	S	0
184	R	1
185	R	1
186	S	1
187	S	1
188	R	1
189	S	1
190	S	1
191	S	1
192	S	1
193	S	1
194	S	1
195	S	1
196	S	1
197	S	0
198	R	1
199	S	1
200	S	1

R - Resistant S – Susceptible

1-presence of polymorphic band
0-absence of polymorphic band

Discussion

5. DISCUSSION

Tomato, *Solanum lycopersicum* L., is one of the most economically important vegetable crops in the world second to potato. Tomato cultivation in the tropics is severely affected by bacterial wilt caused by the soil born pathogen *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. Warm humid tropical climate and the acidic soil conditions prevailing in Kerala favours the incidence of bacterial wilt causing yield loss up to 100 per cent.

As the application of chemicals, soil fumigation and crop rotation are practically ineffective, the use of resistant varieties is the most effective means for control of bacterial wilt. . However, the resistance is unstable because of the wide genetic diversity of the pathogen. Resistance breeding taken up in Kerala Agriculture University, Vellanikkara has resulted in the development and release of the resistant variety Anagha.

Knowledge on the inheritance of resistance is important in breeding for resistance. But the mode of inheritance is still uncertain and the resistance is strongly influenced by environmental conditions such as temperature, pH and moisture content of the soil. Interference of environmental factors hinder the effective selection in segregating population as there may be symptomatic plants in resistant entries and symptomless plants in susceptible entries. DNA marker analysis enables the selection of F_2 plants suitable for use in breeding and genetic analysis. This shows the significance of molecular marker technology in locating genes, controlling bacterial wilt resistance.

Hence a study was conducted on “Molecular markers for bacterial wilt resistance in mapping populations of tomato” with a view to detect trait related markers with special reference to bacterial wilt. The result of evaluation of F_2 population for bacterial wilt resistance in the field, their molecular characterization and detection of trait related markers are discussed here under.

5.1 Development of mapping population

Identified bacterial wilt resistant variety Anagha was crossed with two susceptible genotypes DVRT-1 and Pusa Ruby. The F_1 plants along with the parents were raised in the bacterial wilt sick plot during the period 2006 to 2008. The female parent Anagha was observed to be resistant to bacterial wilt. The resistance to bacterial wilt in Anagha has already been reported by Sadhankumar (1995) and Rajan and Sadhankumar (2002).

Both the F_1 hybrids and male parents i.e., Pusa Ruby and DVRT-1 were observed to be susceptible to bacterial wilt. Hundred per cent susceptible nature of Pusa Ruby has already been reported by Narayanankutty (1985). The susceptibility of F_1 hybrids to bacterial wilt suggests the involvement of recessive genes in Anagha in imparting resistance.While transferring the wilt resistant genes into processing tomatoes Kurian and Peter (2001) and Devi *et al*. (2002) also got F_1 hybrids which were susceptible to bacterial wilt. Sadhankumar (1995) also got susceptible F_1 hybrids while transferring bacterial wilt resistance to fruit crack resistant genotypes in tomato.

Another set of F_1 plants were raised in pots filled with sterilized medium as all the F_1 plants were observed to be susceptible to bacterial wilt. Both the nursery and the seedlings were raised in earthen pots containing sterile soil medium. The medium was sterilized with formaldehyde solution to avoid the inhabiting inoculum of *Ralstonia solanacearum* in the soil. These F_1 plants were selfed to produce F_2 population of both the crosses.

5.2 Evaluation of F_2 population for bacterial wilt resistance in the field

F_2 populations of both the crosses were screened for bacterial wilt in a wilt sick field. Wilting started one week after transplanting, as leaf drooping followed by complete wilting and death of the plant. Bacterial wilt incidence was

confirmed by ooze test. About 90% of the wilted plants showed positive response to bacterial ooze test. Maximum wilt incidence was observed between thirty to forty five days after transplanting. There was no wilting 60 days after transplanting.

Being younger, the seedlings succumbed to wilt more rapidly. The reason for this could be that the seedlings have thinner cortical cells compared to older plants that make the entry of pathogen easier. Reports by Winstead and Kelman (1952) and Celine (1981) also highlighted that wilting was more in juvenile stage as compared to one observed in adult stage.

F_2 population of the two crosses showed different degrees of resistance and susceptibility. Survival rate of the F_2 population of the cross Anagha x Pusa Ruby was more when compared to the cross Anagha x DVRT-1. In the case F_2 population of the cross of Anagha x Pusa Ruby, 32 percentage of F_2 population survived 75 days after transplanting and they were considered to be resistant. But in the case of F_2 population of the cross Anagha x DVRT-1, only 15 percentage of F_2 population survived 75 days after transplanting. So per cent wilt incidence was more for F_2 population of the cross Anagha x DVRT-1 compared to F_2 population of the cross Anagha x Pusa Ruby.

5.3 MOLECULAR CHARACTERIZATION

With the objective of developing trait specific marker for bacterial wilt resistance, molecular characterization of the parent genotypes, F_1 and F_2 population was carried out using RAPD (Random Amplified Polymorphic DNA) assay.

5.3.1 Isolation of genomic DNA

Isolation of good quality DNA without any contamination is very essential for molecular study. Required quantity of DNA should be present to carry out RAPD analysis. DNA was isolated in the morning from tender leaves so as to minimize the interference of polyphenols. The quality and quantity of DNA isolated was best when tender leaves were used as compared to mature and half mature leaf samples (Babu, 2000). Due to the lower content of polyphenols, polysaccharides and other secondary metabolites, which co-precipitate with DNA in the extraction procedure, DNA extraction from plants is preferentially performed from young tissues (Zhang and Mc Stewart, 2000). High amount of these impurities which form co-precipitate with DNA, inhibit DNA digestion and RAPD assay, presumably by irreversible interactions with DNA. 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).

Leaf tissue was ground into paste form using liquid nitrogen. Liquid nitrogen freezes the tissues and helps to prevent nucleic acid degradation and this could be one of the reasons for obtaining intact DNA. Similar observations have been made by Lodhi *et al.* (1994) in grape vine, Sharma *et al.* (2002) in sorghum, chick pea, wheat and soybean and Padmalatha and Prasad (2006) in medicinal and aromatic plants.

Tomato leaf DNA isolation can be hampered by high levels of tannins and polyphenolic compounds. During tissue homogenization, phenolics become oxidized and covalently bind to DNA giving it a brown colour. The irreversible binding produces a gelatinous material, which is hard to separate from organelles and the DNA become unsuitable for amplification and digestion analysis.

During DNA isolation, ß-mercapto ethanol and extraction buffer containing Poly Vinyl Pyrolidone (PVP) were added to overcome the problems

due to high levels of tannins and polyphenolic compounds. ß-mercapto ethanol is a reducing agent, which protect DNA from peroxidase and polyphenol oxidase. ß-mercapto ethanol disrupts the protein disulfide bonds and is thereby capable of initiating protein degradation. PVP complexes with secondary plant products especially polyphenols and tannins by binding them with hydrogen bonds and can be separated from DNA by centrifugation. The use of ß-mercapto ethanol and PVP for overcoming phenolic contamination have already been reported by Nesbit *et al*. (1995) and Padmalatha and Prasad (2006).

The cationic detergent, CTAB (Cetyl trimethylammonium bromide), helps in recovery of relatively pure DNA by helping in lysis of cell membrane and release of nucleic acids and CTAB forms a complex with polysaccharides and prevents co-precipitation of polysaccharides with nucleic acids. On the other hand, it acts as a selective precipitant of nucleic acids in the solution. The DNA is soluble in presence of CTAB at high salt concentration (1.4 M NaCl). In addition, NaCl present in the extraction buffer would also have helped in removal of polysaccharides. The nucleic acids form tight complexes with polysaccharides creating a gelatinous pellet that contains embedded DNA, and polysaccharides (Sharma *et al*., 2002). Certain polysaccharides are also known to inhibit RAPD reactions. Their removal is of great significance in DNA isolation as they distort the results in many analytical applications and lead to many wrong interpretations (Padmalatha and Prasad, 2006).

Extraction buffer also contain EDTA which is a chelating agent and chelates the Mg^{2+} ion which is an essential co-factor for the enzyme to act. It prevents the indigenous endonucleases which act on nucleic acids TE buffer (Tris EDTA) also contain EDTA in which the DNA is dissolved finally.

After the tissue homogenization and addition of extraction buffer, the samples were incubated at 65°C for 15 minutes to accelerate the release of DNA from the nucleus and inactivate DNases and other enzymes that can destroy

DNA.The DNA isolated by Rogers and Bendich (1994) protocol ensure the removal of chlorophyll and other colouring substances such as pigments, dyes etc. by two treatments with chloroform: isoamyl alcohol (24: 1).

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, extracted DNA samples were treated with RNase A. Large amounts of RNA in the sample can chelate Mg^{2+} ions and reduce the yield of polymerase chain reaction (PCR). The contaminating RNA that precipitates along with DNA causes interference with DNA amplification involving random primers and improper priming of DNA templates during thermal cycle amplification. 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).

Protocols given by Chromous Biotech Pvt. Ltd. and Rogers and Bendich (1994) were used for the isolation of DNA. The spectrophotometric readings showed that the quantity of DNA isolated by both the methods was good enough to carryout RAPD assay. The DNA extracted by both the methods were of high quality as it showed an absorbance ratio ranging between1.80 and 1.90 at 260nm/280nm.

5.3.2 RAPD ASSAY

Random Amplified Polymorphic DNA (RAPD) assay is a powerful technique for determining inter and intra-specific DNA variation. Wiliams *et al.* (1990) for the first time demonstrated that single primers of arbitrary sequence can be used to amplify genomic DNA segments and the polymorphism can be detected between the amplified products of different individuals. It is a dominant marker system that is inherited in a Mendelian fashion.

In an RAPD reaction, a single decamer 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. This marker system has been used in many research applications involving the detection of DNA sequence polymorphism, isolation of markers linked to various traits, varietal identification and parentage analysis.

5.3.2.1 RAPD profiling of tomato genotypes

Random decamer primers obtained from Operon Technologies, USA were used for the study. The successful 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*.

Decamer primers (18 number) belonging to OPF, OPAZ, OPAH, OPY, OPS series were used in RAPD assay to detect polymorphism between resistant parent, susceptible parent, F_1, F_2 susceptible bulk and F_2 resistant bulk. In the case of Anagha x Pusa Ruby cross, DNA of Anagha, Pusa Ruby, Anagha x Pusa Ruby F_1, Anagha x Pusa Ruby F_2susceptibe bulk and Anagha x Pusa Ruby F_2 resistant bulk ere used for RAPD assay. In the case of Anagha x DVRT- 1 cross, DNA of Anagha, DVRT- 1 , Anagha x DVRT- 1 F_1, Anagha x DVRT- 1 F_2susceptibe bulk and Anagha x DVRT- 1 F_2 resistant bulk etc were used for RAPD assay. For each primer, ten reactions plus a negative control was set up. The negative control comprised of all the ingredients in the RAPD reaction mixture excluding the template DNA. The negative control was maintained in order to ensure that amplification was due to the tomato genomic DNA alone.

The number of amplification products produced by the primers ranged from two to nine. This difference in amplification could be 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). They have also reported that GC content in the decamer primer influenced the amplification and a GC content of 40 per cent or more in the primer sequence was needed to generate detectable levels of amplified products. The size of the amplicons ranged from 2.51 kb to 95 bp.

Out of 18 primers selected for RAPD assay, 17 primers ie. OPAZ 4,OPAZ 9,OPAZ 16,OPAZ 17,OPAZ 18,OPF 3,OPF6,OPF 9,OPF 16, OPAH 12,OPY 7,OPY8,OPY10,OPS 1,OPS 3,OPS 8,OPS 12 didn't show polymorphism. All the bands produced by these 17 primers were observed to be monomorphic in RAPD profile of both the crosses ie. Anagha X Pusa Ruby and Anagha x DVRT-1. A clear demarcation between the genotypes Anagha and Pusa Ruby with respect to resistance/susceptibility to bacterial wilt was not obtained with the primers screened by Karumannil (2007) in the same lab in the Centre for Plant Biotechnology and Molecular Biology (CPBMB).Lack of polymorphism between resistant and susceptible genotypes, in the case of Tomato Leaf Curl disease was reported by Divakaran (2007).

Though amplification was obtained using 18 primers, polymorphism was produced by only one primer OPS 16, suggesting limited genetic variation in tomato cultivars grown in India. Similar reports also exist in tomato accessions from other regions of the world including both primary and secondary centres of diversity. Existence of very low genetic diversity within cultivated tomatoes could be attributed to self-pollination, artificial selection and founder effect (Archak *et al*., 2002).

A clear demarcation between the genotypes with respect to resistance/susceptibility to bacterial wilt was not obtained with the primers

screened, except the primer OPS16.Polymorphism was produced only between Anagha and DVRT- 1. Using the same primer OPS 16, polymorphism was not there between Anagha and Pusa Ruby. This indicates the need for more number of random primers and more sensitive assay systems to be exploited for discerning the genetic basis of disease reaction in tomato genotypes. Chandrashekhara *et al.* (2003) have also reported the high level of similarity (60-84 %) 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).

Polymorphism was produced by only one primer OPS 16 giving unique band of 1.36 kb in Anagha, Anagha x DVRT-1 F_1, F_2 susceptible bulk, F_2 resistant bulk. That particular band was absent in DVRT-1. Hence, these bands obtained could be the markers contributing resistance to bacterial wilt disease.

Polymorphism was not observed between Anagha and Pusa Ruby with the same primer OPS 16. This could be due to the fact that the Pusa Ruby may contain heterozygous alleles. Since RAPD is a dominant marker, the primer OPS 16 may not be able to produce polymorphism between homozygous dominant (Anagha) and heterozygous (Pusa Ruby) genotypes. This is true in the case of F_1 Anagha x DVRT-1 also. Even though F_1 is susceptible in the field, it produces 1.3 kb band as it is heterozygous and RAPD is a dominant marker.

Since RAPD polymorphisms are the result of either a nucleotide base change that alters the primer binding site, or an insertion or deletion within the amplified region, polymorphisms are usually noted by the presence or absence of an amplification product from a single locus (Williams *et al.*, 1990).

5.3.2.2 Analysis of the mapping population with OPS 16

Though amplification was obtained using 18 primers, polymorphism was produced by only one primer OPS 16. Polymorphism was observed only between Anagha and DVRT- 1, when RAPD assay was done with the primer OPS 16. Using the same primer OPS 16, polymorphism was not there between Anagha and Pusa Ruby. So further RAPD analysis of segregation pattern of F_2 population was carried out in F_2 population of Anagha X DVRT -1 only. Mapping population of Anagha x Pusa Ruby were not analyzed individually with OPS 16 primer.

When the 157 plants of the F_2 population of the cross Anagha x DVRT-1 were grown in a bacterial sick field, 27 plants were found to be resistant to bacterial wilt and 130 plants were susceptible. On RAPD assay with the primer OPS 16, all the 27 resistant plants showed 1.36 kb polymorphic band. Out of 130 susceptible plants, DNA of 95 plants showed this band and the remaining 35 plants didn't show the 1.3 kb band.

The 27 resistant plants produced the 1.36 kb polymorphic band because the resistant gene for bacterial wilt resistance was present in homozygous recessive condition in them. The 1.3 kb band was absent in the DNA of 35 susceptible plants as these plants were having resistant genes in homozygous dominant state. DNA of the 95 susceptible plants produced 1.36 kb band because they had the resistant gene in heterozygous condition. Such a condition can occur only when the gene for resistance is recessive in nature. The recessive nature of the resistant gene is evident from the fact that all the F_1 plants succumbed to bacterial wilt, when they were grown in bacterial wilt sick plot.

All this suggests that the gene(s) for resistance to bacterial wilt in Anagha is recessive in nature. This can be confirmed only when further generations are studied. By observing field reaction and RAPD assay of further generation, we

can infer whether the polymorphism given by the primer OPS16 is related to bacterial wilt resistance or not. Production of backcross population or F_3 population of Anagha x DVRT-1 would be the next step for confirming the relation of bacterial wilt resistance to the polymorphic band produced by the primer OPS 16.

Summary

6. SUMMARY

The investigation on 'Molecular markers for bacterial wilt resistance in mapping populations of tomato' were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Olericulture, College of Horticulture, Vellanikkara during the period 2006 to 2008. The main objective of the study was to detect and characterize trait related markers with special reference to bacterial wilt. Three tomato genotypes viz. Anagha (resistant to bacterial wilt), DVRT-1 (susceptible to bacterial wilt) and Pusa Ruby (susceptible to bacterial wilt) were used for the study.

The salient findings of the study are as follows:

1) Bacterial wilt resistant variety Anagha was crossed with two bacterial wilt susceptible varieties viz. DVRT-1 and Pusa Ruby to develop F_1 population. F_1 plants of both the crosses Anagha X DVRT - 1 and Anagha X Pusa Ruby were grown in bacterial sick field and all the F_1 plants were observed to be susceptible to bacterial wilt.

2) F_1 plants were raised in sterilized medium also and they were selfed to produce the F_2 mapping population.

3) F_2 populations of both the crosses were grown in a wilt sick field. DNA was isolated from all the individually labelled F_2 plants.

4) F_2 plants were classified into three groups
   a) Wilted due to bacterial wilt(confirmed by ooze test)
   b) Wilted due to other reasons(no ooze)
   c) Resistant to bacterial wilt

5) Among the 2 crosses evaluated, wilt incidence was more for the cross Anagha x DVRT-1. In F_2 population of the cross Anagha x DVRT-1, out

of the 200 plants evaluated, 28 plants were found to be resistant to bacterial wilt and 157 plants were susceptible. Plants wilted for reasons other than bacterial wilt were 15 in number. In the F_2 population of the cross Anagha x Pusa Ruby out of 200 seedlings transplanted, 62 plants were found to be resistant to bacterial wilt and 130 plants were susceptible. Plants wilted for reasons other than bacterial wilt were eight in number.

6) The protocols suggested by and Rogers and Bendich (1994) and kit from Chromous Biotech Pvt Ltd.Banglore were tried for DNA isolation. RNAse treatment was given to those DNA isolated using Rogers and Bendich protocol to remove RNA contamination.

7) The quality and quantity of DNA isolated by both the methods were ensured by nanodrop spectrophotometer and it indicated relatively pure DNA in the samples free from RNA and other contaminants like protein and other pigments.

8) During isolation DNA from 28 plant samples of F_2 population of the cross Anagha x DVRT-1 and 31 DNA samples of F_2 population of the cross Anagha x Pusa Ruby were degraded. Degraded DNAs and DNA from plants wilted due to reasons other than bacterial wilt were discarded. So further study was done on 157 DNA of F_2 population of the cross Anagha x DVRT- 1 and 160 DNA of F_2 population of the cross Anagha x Pusa Ruby. In F_2 population of the cross Anagha x DVRT-1,observation on the incidence of bacterial wilt after the removal of degraded DNA and DNA of plants wilted due to reasons other than bacterial wilt were as follows
   a) Wilted due to bacterial wilt(confirmed by ooze test) – 130 plants
   b) Resistant to bacterial wilt – 27 plants

In F_2 population of the cross Anagha x Pusa Ruby

a) Wilted due to bacterial wilt(confirmed by ooze test) – 110 plants
b) Resistant to bacterial wilt – 50 plants

9) Based on the RAPD assay done in Sol Genome project with Anagha, Pusa Ruby and DVRT-1, with different primers from OPBG, OPF, OPAZ, OPY, OPS, OPA, OPN .OPAH series; 18 primers were selected for further studies.

10) Based on the field reaction of F_2 population from both the crosses, the DNA isolated from 20 susceptible plants were pooled to make F_2 susceptible bulk/susceptible pool. DNA isolated from the 20 F_2 plants showing bacterial wilt resistance were also pooled and made F_2 resistant bulk.

11) The 5 genotypes i.e. resistant parent, susceptible parent, F_1, F_2 susceptible bulk and F_2 resistant bulk of the two crosses differing in reaction to bacterial wilt disease was characterized using 18 selected primers from OPF, OPAZ, OPS, OPAH, OPY series.

12) Though the amplification was there with all the 18 primers, polymorphism was produced by only one primer OPS 16 between Anagha and DVRT -1, suggesting limited genetic variation in tomato cultivars grown in India.

13) Using the same primer OPS 16, polymorphism was not there between Anagha and Pusa Ruby. This could be due to the fact that the Pusa Ruby may contain heterozygous alleles. Since RAPD is a dominant marker, the primer OPS 16 may not be able to produce polymorphism between homozygous dominant (Anagha) and heterozygous (Pusa Ruby) genotypes. So further study of segregation of F_2 population was carried out only in F_2 population of Anagha X DVRT -1 only.

14) RAPD assay could not identify the specific marker for resistance/susceptibility to bacterial wilt disease except with OPS 16 primer, which gave a unique band of size ~1.3 kb in Anagha, Anagha X DVRT-1 F_1, Anagha XDVRT-1 F_2 susceptible bulk and Anagha X DVRT-1 F_2 resistant bulk, but was absent in DVRT-1.

15) When F_2 population (157) of the cross Anagha x DVRT-1 was analyzed individually with the primer OPS 16, DNA of all the 27 resistant plants showed 1.36 kb band. Out of 130 susceptible plants, DNA of 95 plants showed this band and the remaining 35 plants didn't show the 1.3 kb band.

16) The 27 resistant plants produced the 1.36 kb polymorphic band because the resistant gene for bacterial wilt resistance was present in homozygous recessive condition. The 1.3 kb band was absent in the DNA of 35 susceptible plants as these plants were having resistant genes homozygous dominant condition in them. DNA of the 95 susceptible plants produced 1.3 kb band because they had the resistant gene in heterozygous condition. Such a condition can occur only when the gene for resistance is recessive in nature.

17) The recessive nature of the resistant gene is also evident from the fact that all the F_1 plants succumbed to bacterial wilt, when they were grown in bacterial wilt sick plot.

18) When the whole F_2 population of the cross Anagha x DVRT-1 was analysed individually using RAPD assay with OPS 16 primer, a segregation of polymorphic band was obtained. All this suggests that the gene for resistance to bacterial wilt in Anagha is recessive in nature and the polymorphic band produced by the primer OPS 16 could be related to bacterial wilt resistance.

19) This can be confirmed only by analyzing backcross population or F_3 population of Anagha x DVRT-1. By observing field reaction and RAPD assay of F_3 population or backcross population, we can infer, whether the polymorphism given by the primer OPS 16 is related to bacterial wilt resistance or not.

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Appendices

APPENDIX-I

Composition of Reagents Used for DNA Isolation

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-II

Composition of Buffers and Dyes used for gel electrophoresis

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

MOLECULAR MARKERS FOR BACTERIAL WILT RESISTANCE IN MAPPING POPULATIONS OF TOMATO

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The investigation on "Molecular markers for bacterial wilt resistance in mapping populations of tomato" was undertaken at the Centre for Plant Biotechnology and Molecular Biology and Department of Olericulture, College of Horticulture, Vellanikkara during the period 2006-2008 to detect trait related markers linked to bacterial wilt resistance. Anagha was used as the resistant parent. DVRT-1 and Pusa Ruby were used as the susceptible parents.F_1 plants of both the crosses were found to be susceptible when grown in wilt sick field. F_1 plants were raised in sterile media in pots and were selfed to produce F_2 population of both the crosses.

F_2 populations were raised in a bacterial wilt sick plot. F_2 population of both the crosses, after transplanting into bacterial wilt sick plot, showed wilt symptoms. The symptom started as leaf drooping followed by complete wilting and death of the plant. Bacterial ooze test was performed to confirm the infection by *Ralstonia solanacearum*. Among the two crosses evaluated, wilt incidence was more in F_2 population of the cross Anagha x DVRT-1. In the F_2 population of the cross Anagha x DVRT-1,out of the 200 plants ,28 plants were observed to be resistant to bacterial wilt and 157 plants were found to be susceptible ,confirmed with ooze test and 15 plants wilted for reasons other than bacterial wilt.

In the F_2 population of the cross Anagha x Pusa Ruby, out of the 200 seedlings transplanted, 62 were observed to be resistant to bacterial wilt and 130 plants were found to be susceptible. Plants wilted for reasons other than bacterial wilt were eight in number. Genomic DNA of parents, F_1 and F_2 populations was isolated, purified and subjected to RAPD assay for molecular characterization. The protocols suggested by Rogers and Bendich (1994) and kit from Chromous Biotech Pvt Ltd. were used for DNA isolation.

The five genotypes i.e. resistant parents, susceptible parent, F_1, F_2 susceptible bulk and F_2 resistant bulk, differing in reaction to bacterial wilt disease were characterized using 18 selected primers from OPF, OPAZ, OPS, OPAH, and OPY series. Though amplification was obtained with all the 18 primers, polymorphism was produced by only one primer OPS 16, in only one cross i.e. Anagha X DVRT-1.RAPD assay could not identify the specific marker for resistance/susceptibility to bacterial wilt disease except with OPS 16 primer, which gave a unique band of size ~1.3 kb in Anagha, Anagha X DVRT-1 F_1, Anagha XDVRT-1 F_2 susceptible bulk and Anagha X DVRT-1 F_2 resistant bulk, but was absent in DVRT-1.Polymorphism was not observed between Anagha and Pusa Ruby in the RAPD assay done with the 18 selected primers. So further study of segregation of F_2 population was carried out only in F_2 population of Anagha X DVRT -1.

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Whole F_2 population of the cross Anagha x DVRT-1 was analyzed individually with the primer OPS 16. DNA of all the resistant plants showed 1.36 kb band. In the case of susceptible plants, a segregation of polymorphic band was observed. All this suggests that the gene for resistance to bacterial wilt in Anagha is recessive in nature and the polymorphic band produced by the primer OPS 16 could be related to bacterial wilt resistance.

The recessive nature of the resistant gene is also evident from the fact that all the F_1 plants succumbed to bacterial wilt, when they were grown in bacterial wilt sick plot. All these can be confirmed only by analyzing backcross population or F_3 population of Anagha x DVRT-1.So future line of work is to develop backcross population or F_3 population, and detect whether the polymorphic band given by the primer OPS 16 is related to bacterial wilt resistance or not.