INTEGRATION OF COMBINED DISEASE RESISTANCE FOR BACTERIAL WILT AND ToLCV IN TOMATO (*Solanum lycopersicum* L.) THROUGH MARKER ASSISTED SELECTION

By

BELGE SHRIRAM ASHRU

(2012-11-105)

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

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THESIS

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2014

DECLARATION

I hereby declare that the thesis entitled **"Integration of combined disease resistance for bacterial wilt and ToLCV in tomato (*Solanum lycopersicum* L.) through marker assisted selection."** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara **Belge Shriram Ashru**
Date : 16/09/2014 (2012-11-105)

CERTIFICATE

Certified that the thesis entitled **"Integration of combined disease resistance for bacterial wilt and ToLCV in tomato (*Solanum lycopersicum* L.) through marker assisted selection."** is a record of research work done independently by **Mr. Belge Shriram Ashru** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to him.

Vellanikkara
Date: 16/09/2014

Dr. P. A. Nazeem
(Chairman, Advisory Committee)
Professor and Co-ordinator (DIC)
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture,
Vellanikkara, Thrissur

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Belge Shriram Ashru** a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology,** agree that the thesis entitled **"Integration of combined disease resistance for bacterial wilt and ToLCV in tomato (*Solanum lycopersicum* L.) through marker assisted selection."** may be submitted by **Mr. Belge Shriram Ashru** in partial fulfilment of the requirement for the degree.

Dr. P. A. Nazeem
(Chairman, Advisory Committee)
Professor and co-ordinator (DIC)
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture,
Vellanikkara, Thrissur.

Dr. P. A. Valsala
(Member, Advisory committee)
Professor and Head
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

Dr. Deepu Mathew
(Member, Advisory committee)
Assistant Professor
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

Dr. R. Sujatha
(Member, Advisory committee)
Associate Professor
Department of Biotechnology
College of Agriculture
Padannakkad

Dr. S. Nirmala Devi
(Member, Advisory committee)
Professor
Department of Olericulture
College of Horticulture
Vellanikkara, Thrissur

External examiner
Dr. R. Keshavachandran
Professor and Co-ordinator (Retd.)

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Belge Shriram Ashru

Dedicated to my beloved
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ABBREVIATIONS

%	Percentage
>	Greater than
µg	Microgram
µl	Microlitre
A	Ampere
AFLP	Amplified Fragment Length Polymorphism
BGMV	Bean golden mosaic virus
Bp	Base pair
CI `	Coefficient of infection
cm	Centimetre
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EST	Expressed sequence tags
g	Gram
GD	Genetic Distance
ha	Hectare
ISSR	Inter Simple Sequence Repeat
kb	Kilo basepairs
L	Litre
M	Molar
MAS	Marker Assisted Selection
mg	Milligram
ml	Millilitre
mM	Milli mole
NBPGR	National Bureau of Plant Genetic Resources

ng	Nanogram
^{o}C	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
PDI	Per cent disease incidence
PDS	Per cent disease severity
P^{H}	Hydrogen ion concentration
PVP	Poly vinyl pyrolidone
QTL	Quantitative trait loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RILs	Recombinant Inbred Lines
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SCAR	Sequence Characterized Amplified Region
SSR	Simple Sequence Repeats
STMS	Sequence Tagged Microsatellite Sites
STR	Short Tandem Repeat
STS	Sequence Tagged Sites
TAE	Tris Acetate EDTA
TE	Tris EDTA
TZC	Tetrazolium chloride
U	Unit
UV	Ultra violet
V	Volts
β	Beta

Introduction

1. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an important and most widely grown vegetable crop of both tropics and sub tropics of the world, belonging to the family *Solanaceae* and ranks second in importance among vegetables. It is grown for its edible fruit, which can be consumed, either raw or cooked or in the form of various processed products like juice, ketchup, sauce, pickle, pastes, puree and powder. It is esteemed as an important source of vitamins A and C. The genus *Solanum* consists of nine closely related species. Tomato cultivation has become increasingly popular since the mid-nineteenth century because of its varied climatic change and high nutritive value. It also contains lycopene and beta-carotene, which are anti-oxidants that promote good health. The high demand for tomato makes it a high value crop that can generate much income to farmer. The main tomato growing countries in the world are China, USA, India, Turkey, Italy, Iran, Egypt, Brazil, Spain and Mexico.

In India, it occupies an area of about 0.88 million hectares with the production of 182.2 metric tonnes and productivity 20.7 metric tonnes (www.nhb.gov.in). Though, the area under tomato cultivation is high, the productivity is low, attributed to the potential loss in yield due to a number of diseases.

The area under tomato cultivation in Kerala is very meagre. The main limiting factor is the incidence of bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi *et al*., 1995). Symptoms of the disease include rapid and complete wilting of grown up plants. Pathogen is mostly confined to the vascular region. Upon infection, bacterial polysaccharides mechanically block the vascular system and check the translocation of water and other food material resulting in wilting of plants. The warm humid tropical climate and acidic soil conditions favour the incidence of this disease in Kerala. Crop loss upto 100 per cent is reported due to bacterial wilt (Yadav, 2011). This disease is wide spread in Maharashtra, Karnataka, Orissa, Himachal Pradesh, Jharkhand and West Bengal. The pathogen

is soil borne and survives at least for two years even in the absence of any host (Shekhawat *et al*., 1979). Due to the soil born nature of the pathogen, chemical control measures have not been successful in controlling this disease. Use of resistant varieties is the obvious method to tackle this problem.

Resistance breeding taken up in Kerala Agriculture University has so far produced three bacterial wilt resistant varieties *viz*., Sakthi, Mukthi, Anagha and one tolerant variety Vellayani Vijay. These varieties are however susceptible to another serious disease called Tomato leaf curl virus (ToLCV) necessitating the development of varieties with combined resistance. Tomato leaf curl virus (ToLCV) disease is one of the most serious diseases of tomato in the Indian sub-continent and many other tropical and subtropical Asian countries (Yadav, 2011). This disease is caused by a Gemini virus transmitted by the whitefly *Bemisia tabaci* (Anbinder *et al*., 2009). The affected plant exhibit curling, puckering, reduction in leaflet size, severe stunting and reduction in fruit set. However, severely infected young plants almost fail to produce any fruit. This disease can cause yield losses up to 99-100 per cent (Singh and Sadashiva, 2007). Chemical control measures as well as integrated pest management (IPM) strategies employed for controlling the vector have not been successful in controlling the disease. Under these circumstances, breeding for combined resistance through marker assisted selection appears to be a promising and eco-friendly approach for controlling the disease. Sakthi was selected as the female parent and its special features were bacterial wilt resistance, semi determinate growth habit, green shoulder and flat round fruit. IIHR 2196 was selected as male parent and its special features include resistance to ToLCV, high yield with slightly cylindrical fruit shape.

In disease resistance breeding, breeders frequently counter various interactions among the resistance genes confusing selection through conventional breeding. These problems can be overcome by identifying specific molecular marker linked to disease resistance genes and tracing the inheritance pattern of the

molecular marker in breeding programme through marker assisted selection (MAS).

DNA marker technology has been used in plant breeding programmes since early 1990s. It simplifies the screening for specific traits that are highly complex provided a closely linked marker to the phenotype is identified. Now days, large numbers of molecular markers are reported for specific disease but only few are found significant in marker assisted selection (Foolad and Panthee, 2012).

The present study entitled “Integration of combined disease resistance for bacterial wilt and ToLCV in tomato (*Solanum lycopersicum* L.) through marker assisted selection” was undertaken as part of International Sol Genome Project with an objective to investigate the inheritance pattern of the molecular markers identified and integrate combined disease resistance to bacterial wilt and ToLCV in tomato through marker assisted selection.

Review of Literature

2. REVIEW OF LITERATURE

Among the many bacterial and viral diseases affecting tomato crop, the bacterial wilt caused by *Ralstonia solanacearum* and tomato leaf curl virus (Geminivirus) are the most important ones (Anonymous, 1998). Worldwide occurrences of these pathogens already have been detected. Extensive host range including hundreds of plant species makes them the most important bacterial and viral diseases (Buddenhagen and Kelman, 1964; Hayward, 1964). Because of the highly diversified nature of pathogen, it has formed heterogeneous groups of related strains (Fegan and Prior, 2005; Hayward, 1994). Therefore, rapid and highly sensitive detection methods are required to reduce field losses and limit the spread of bacterial wilt and ToLCV.

A brief review on various aspects of bacterial wilt and ToLCV in tomato is presented in this chapter.

2.1 Origin and history of tomato

Scientific information indicates that the cultivated tomato has originated in a wild form in the Peru. The distribution of the allele studied at the genetic basis also gives evidence of the origin of cultivated tomato in Peru (Rick, 1969).

According to Jenkins (1948), Mexico, particularly the Veracruz-Puebla area, is the centre of varietal diversity of cultivated tomato. It might have been the source of cultivated tomato of the old world, on the basis of archaeological and historical evidence. The cultivated type of South America also might have been introduced from Mexico. Thus, this might have been the centre of domestication (Rick, 1976).

Solanum lycopersicum L., the wild cherry tomato, has been found in Mexico, most commonly at wild places, borders of fields, road-cut or stream-bank, where there are suitable conditions for tomato growing. This has been found in most of the tropical places (Jenkin, 1948). Even the wild types behave as

introduced plants and do not seem to have originated from this place. A large group of diversity existing in Mexico may be the mutant of the original types. A critical analysis indicates that the cultivated tomato, presumably, might have been carried from the slopes of the Central America by the Indians at the time of their migration. The allogamy variation in cultivated and closely related species of tomato indicates the hypothetical sequence: var. cerasiforme domestication (Mexico) - cultivars in Europe (Rick and Fobes, 1975).

The biosystematics studies in *Lycopersicum* and closely related species of *Solanum* by Rick (1979) suggest that the species of *Lycopersicum* have been evolved via gene substitution. According to Alpatev (1981) the cultivated tomato has originated as a result of spontaneous and artificial hybridization between plants of different botanical varieties and artificial selection by man Rick (1976).

The first known record of tomato is the year 1554 and after 200 years it has gradually spread to other part of the world. The Italians grew it in about 1550 and might have been the first Europeans to eat it. After 25 years, it was introduced to the English, Spanish and mid-European gardens as a curiosity. In the USA, the plant was first grown by Thomas Jefferson in Virginia in 1781. Later it was spread to Philadelphia by the French refugees in 1789 and to Massachusetts in 1802 by an Italian painter. Since1800, tomatoes are being used as food (Boswell, 1949). Now there has been a tremendous improvement in this crop and it has become a major source of vitamins and minerals. It also contains lycopene and beta-carotene, which are anti-oxidants that promote good health. The details of the history of the use of tomato have been given by McCue (1952).

2.2 Botany

Tomato belongs to the genus *Solanum* of the family solanaceae. This genus includes many species including some polymorphic types. Usually it is herbaceous, annual to perennial, prostrate and sexually propagated. Occasionally, however, it is also asexually propagated.

Tomato produces a strong tap root but very often it is damaged at the time of transplanting. The adventitious roots develop at the rate of about 33 mm per day penetrating 1 m depth and attaining a length of 1.5 m (Ward, 1964).

The stem is soft, brittle, and hairy when young and hard, woody, and copiously branched when mature. Its organisation is in the sequence of two or three-leaved symposia. The plant can be classified into three: groups on the basis of their growth habit.

Determinate: The development of the growing point is ceased; the plants are upright; the stem is short without a side-shoot; and the leaves are thick dark green.

Indeterminate: The plant grows this continued and there is less initiation of flowers and fruit on the stem. The lateral buds always exist to continue vegetative growth.

Semi-determinate: The plant growth habit is in between determinate and indeterminate (PPV and FRA).

The leaf is alternate, petiolate, 2-15 in wide; the leaflets are unequal odd pinnate; the apex is narrowed or accumulate, acute, irregularly serrate. The number of leaflets varies from four to several and the length of the leaf from 6 to 12 in. On the several leaves, hair or trichomes are formed by the outer growth of the epidermal cells (Flores and Espinoz, 1977).

The fruit is soft berry. Glandular hairs and glands are usually present on the small fruit which degenerate in the advanced stage. The fruits of some varieties or species do not have glandular hair and gland. The tomato fruits have number of fruit shapes, such as flattened, slightly flattened, circular, rectangular, cylindrical, heart shaped, ovoid, pear shaped (PPV and FRA, 2006). In the fruit there are five main parts of the fruit- walls (outer and inner), skin, locular, pulp, and seed. The quality of fruit depends on the pericarp.

2.3 Genetics

It was Winkler (1909), who has first reported that the chromosomes in tomato, are 24 (somatic) and 12 (haploid). This finding has been confirmed by several other workers. Twelve is considered as basic chromosome number since there is no pairing of the chromosomes at zygotene in haploids (Humphrey, 1934).

Barton (1950) has very critically ascribed the differentiated structure of all the chromosomes and numbered them on the basis of their size. The chromosomes are identified on the basis of the relative length of the differentiated segments, the arm ratio, and the position of the primary and secondary constrictions.

Haploids in comparison with diploids are morphologically smaller, spread less, and grow luxuriantly. Their flowers are distinctly smaller and higher sterile. Haploids are major sources of homozygous diploids and such plants are produced by decapitating young haploid plants which is known as asexual method of diploid production (Lindstrom, 1941). Haploid can be selected for low temperature tolerance from microspores (Zamir *et al*., 1982).

Triploids are produced from the progenies of diploid, tetraploids, crosses of tetraploids and diploid, and grafts. Such plants originate from the fusion of diploid and haploid gametes (Lesley, 1928). Triploids have been reported to originate as adventitious shoots from the callus of a tomato stock grafted with a scion of *Solanum luteum* (Huskins, 1934).

Winkler (1916) has first reported autotetraploids in the adventitious shoot of tomato scion grafted upon the *Solanum nigrum* stock. Generally tetraploids are produced by immersing the actively growing point of small seedling in 0.2 to 0.5 colchicine solution for 10-15 h. Tetraploids exhibit 48 chromosomes at metaphase of mitosis. At diakinesis of meiosis mostly quadrivalents are discernible. The frequency of quadrivalents is not constant. Sometimes all 48 chromosomes form quadrivalents (Lindstrom and Humphrey, 1932).

2.3.1 Linkage

Sometimes characters are associated in the inheritance due to the location of genes in proximity on the same chromosome. Linkage is caused by the linked genes being carried on the same chromosome.

Aneuploidy has been used extensively to determine the linkage groups on the chromosomes (Rick and Khush, 1969). It is essential to know the proper position of the gene on chromosome for mapping. Linkage tester stocks marking each chromosome or chromosome arm have been used (Rick and Zobel, 1972).

Several genes have thus been located on various chromosomes (Jones, 1917; Lindstrom, 1941; Butler, 1951; Khush and Rick, 1967; Rick, 1982).

The first linkage group studied by MacArthur (1923) consist of four pair of genes. The characters of broad leaf and macro calyx have been used as markers for linkage group 11 and 12, respectively (Butler, 1951).

Later on Barton *et al*., (1955) have developed more interest for genetic studied of tomato and formulated certain rules for the nomenclature of tomato linkage studies. The important rules such as, the chromosomes are numbered according to their length at pachytene; the linkage group continues to be designated by Roman numerals and chromosomes by Arabic numbers; the mutant genes are designated by letter symbols, the translocation are designed as "T", inversion by "*In*", deficiencies by "*Df*" etc. they have listed 107 gene symbols along with their synonyms and main phenotypic characters.

Rick (1971) reported 233 genes assigned to the chromosomes. The linkage maps are presented in which the precise location of 300 genes are displayed (Rick, 1982).The tight linkages observed can be exploited for marker-based selection of desirable genes in breeding programs (Tanksley *et al.*, 1992). This level of marker saturation offers new opportunities for genome research in tomato and potato reveals a number of interesting features of chromosome structure and evolution

that were not apparent from previous linkage maps of the tomato/potato genomes (Bertrand *et al*., 2007).

2.4 Bacterial wilt and its significance

2.4.1 Pathogen

Bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi *et al*., 1995) which is a soil borne pathogen. It can survive in the soil upto two years without any host. Due to the wide range of the pathogen it is very difficult to control the pathogen by any chemical methods. Host plant resistance is the obvious method to tackle this problem and environmentally safe, with low running costs.

The first report on bacterial wilt of tomato in India was by Hedayathulla and Saha, (1941).

Tahat and Sijam, (2010) classified *Ralstonia solanacearum* as of Kingdom: Bacteria, Phylum: Proteobacteria, Class: Beta, Order: Burkholderiales, Family: Ralstoniaceae and Genus: Ralstonia.

Hayward (1964) classified *Ralstonia solanacearum* into biotype 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 reaction.

Smith (1996) published the first description of *Ralstonia solanacearum* which causes a wilt disease of solanaceous plants.

Ralstonia solanacearum is a complex pathogen, differing in host range and pathogenicity. Geographical variation occurs in the organism. Buddenhagen *et al*., (1962) classified *Ralstonia solanacearum* isolates from a wide range of host in Central and South America, based on host range, pathogenicity and colony appearance on TZC medium, into 3 races i.e., race 1, race 2. And race 3. Race 1

Race 1 (Solanaceous strain) – It has wide host range, distributed throughout the lowlands of tropic and subtropics. They attack tomato, tobacco and many solanaceous and other weeds.

Race 2 (Musaceous strain) – This is restricted to *Musa* spp. and few perennials hosts initially limited to American tropic and spreading to Asia.

Race 3 (Potato strain) – This is restricted to potato and few alternate hosts in tropics and subtropics.

2.4.2 Ecology of the pathogen

The ecology of the pathogen in infested soil is poorly understood. It is inferred that the primary inoculum came from the soil but there was no conclusive evidence that the pathogen is a ubiquitous inhabitant in the soil (Buddenhagen and Kelman, 1964). Under natural conditions, the pathogen was able to survive saprophytically in the soil for as long six years (Chester, 1950).

Pseudomonas solanacearum does not survive in the soil for prolonged periods because it is not a strong competitor. It does not survive in the soil itself but survive on or in plant roots. The bacterium appears to survive by continually infecting the roots of susceptible or carrier plants or by colonising the rhizosphere of non-host plants (Gilbert, 2002). Survival of *Ralstonia solanacearum* in the rhizosphere has been documented by Gilbert, (2002) who reported that the bacterium invades the roots of presumed non-hosts such as bean and maize. Long term survival was associated with localised or systemic infection of plants that did not express symptoms of bacterial wilt.

2.4.3 Symptomology

Walker (1952) reported that the first expression of the disease is wilting of the lower leaves of the plants and it leads to the entire wilting of the plants. Dwarfing or stunting of the plants 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 (Chupp and Sherf, 1960). Hildebrant (1950) reported the entry of the bacterium through natural opening of the plant. Chupp and Sherf (1960) reported that the bacteria can enter at the point of origin of secondary roots. The roots and the lower part 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 (Gilbert, 2002).

According to Hussain and Kelman (1957), breakdown of plant tissue by the pathogen is due to the cellulose and polygalacturonase enzyme produced by the pathogen. Stop tissues growth and plugging finally result in the death of the plant.

Visible symptoms of the disease occur within 2 to 8 days after the entry of the pathogen into the host plant (Kelman, 1953; Chupp and Sherf, 1960). The pathogen first enters into the intercellular spaces of cortex. From there, it moves to pith and xylem vessels.

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

2.4.4 Disease cycle and epidemiology

Ralstonia solanacearum is a soil borne and waterborne pathogen; which can survive and disperse for various periods of time in infested soil or water, which can form a reservoir source of inoculum. The bacterium usually infects tomato plants through the roots (through wound or at the point of emergence of lateral roots). Soil borne organisms, such as the root knot nematode, can cause injury to plant roots and favour penetration of the bacterium.

The source of inoculum can be infected potatoes (seed tubers, harvest leftover and infected plants) or infested soil, or both. Anonymouse, (1998) reported the pathogen can survive in soil (mostly on plant debris) and in the rooting system and rhizosphere of many hosts (weeds and other host crops). Plant infection can occur through stem injuries caused by cultural practices or insect damage. In some cases, plant-to-plant spread can occur when bacteria move from roots of the infected plants to roots of nearby healthy plants, often via irrigation practices. Spread of bacteria by aerial means and subsequent plant contamination through foliage is not known to occur, thus making *R. solanacearum* a non-airborne pathogen. High temperatures (29-35^{o} C) play major role in pathogen growth and disease development. Several other factors that may affect pathogen survival in soil and water may also favour disease development, including soil type and structure, soil moisture content, organic matter in soil, water pH, salt content and the presence of antagonist microorganism.

R. solanacearum can survive for days to years in infected plant materials in soil, infected surface, irrigation water and infected weeds. From these sources of inoculum, bacteria can spread from infected to healthy field by soil transfer on machinery and surface runoff water after irrigation or rainfall.

At low temperatures (<4^{o} C) bacterial population density fall rapidly but the bacteria still can survive, often in a physiological latent state. Bacteria were shown to be increasingly released from semi-aquatic weeds after winter when temperatures start to increase.

2.4.5 Morphological characteristics of the pathogen

Ralstonia solanacearum is gram negative, rod shaped, and measuring 1.5-2 x 0.5-0.7µm in size, motile and aerobic. But under some circumstances, it grows anaerobically in media containing nitrate and an appropriate carbon source. It can be grown in agar medium, tyrosine medium or in potato and slime production on peptone beef extract agar medium (Nayar, 1982). The isolated colonies shows circular, smooth, raised, creamish white colonies with pink centre and convex

with entire margin. Biovar V type isolates are produced irregular round, rough, flat, creamish white with pink centre colonies (Mathew, 2001).

Optimum temperature for growth of the isolates varies from 30-35^{o} C and temperature above 45^{o} C is lethal to the pathogen. The pH requirement for the growth is 5.5-7.0. The pathogen loses its virulence very rapidly in culture due to transformation to avirulent mutants and the virulence is retained by preserving the culture in sterile distilled water at room temperature (22-25^{o}C) or under refrigerated condition (4^{o}C) and in 20 per cent glycol at -80^{o}C for long term storage. Among the different methods of inoculation, leaf clipping (Mathew, 2001), root dipping and pseudo stem inoculation (Sambasivam, 2003) are the best method. For the artificial inoculation of the pathogen, fresh bacterial ooze suspension of OD_{600nm} = 0.3 is the best inoculums to ensure uniform, maximum, and rapid development of wilt symptoms rather than cultural suspension.

2.4.6 Pathogenicity test for bacterial identification

The root severing and root drenching method were used to inoculate Capsicum plants with *R. solanacearum* (Wang and Berke, 1997). The injury was made to roots of 28 days old seedling and 30 ml bacterial suspension was inoculated into each pot (Wang and Berke, 1997). In pathogenicity test of *R. solanacearum* on *Moringa oleifera*, inoculation of bacterium was performed by spraying the bacterial suspension onto pin pricked leaf axil of healthy plants and by dipping the cut ends of roots of the healthy plants in bacterial suspension (Estelitta *et al.*, 1997). It was observed that wilting symptoms in plants has been developed after 10 to 20 days of inoculation. Vudhivanich (1997) has used micropipette technique for injection of various concentrations of *R. solanacearum* inoculums directly into the tomato plant by inserting diagonally into the stem at the third leaf axil from the top.

2.4.7 Control measures for *R. solanacearum*

Several control measures have been described to control the disease but not a single method provides complete control of bacterial wilt (Melton *et al.*, 2004). Therefore, for effective management of disease, different strategies like crop rotation, nematode management, chemical application, resistant cultivar and proper cultivation, stalk and root destruction after harvest should be used efficiently (Lucas, 1975; Shew and Lucas, 1991; Fortnum and Martin, 1998). The effects of soil temperature on resistance of tomato cultivars to bacterial wilt were studied. The rate of development of wilt in plants is found affected by different cultivar and soil temperature (Mew and Ho, 1977).

2.5 Tomato Leaf Curl Virus (ToLCV) and its significance

ToLCV is reported to be a serious disease on tomato throughout India. Each year this disease reduced the yield of tomato crops all over the world. Sastry and Singh (1973) reported that ToLCV infested plants produced very few fruits when infested within 20 days after transplanting and resulted up to 92.30 per cent yield losses.

Banerjee and Kalloo (1987) reported that the major constraint in the cultivation of tomato was the outbreak of ToLCV during summer in south India and autumn in north India. Sadashiva *et al*., (2007) reported that incidence of the disease results in yield loss between 70 to 100 per cent.

2.5.1 Taxonomy

Tomato leaf curl virus disease (ToLCV) is caused by geminivirus and is transmitted by whitefly (*Bemisia tabaci*) belonging to family geminiviridae and genus begmovirus (Anbinder *et al*., 2009). The disease occurrence is correlated with size of the *B. tabaci* population and attributed to the failure of the crop. Several weed species occurring in nature are known to be hosts for both virus and

the vector and reported to be major contributors of ToLCV inoculum for the disease outbreak (Gameel, 1977).

Geminiviruses are plant viruses that belong to the family Geminiviridae, first described by Goodman in 1977. Geminiviruses are characterised by the unique Gemini shape of a fused icosahedral viral particle. The family Geminiviridae is comprised of three genera, all of which share similarities in genome organisation, insect transmission and host rang.

Viruses were having monopartite and bipartite genomes. Begomoviruses are transmitted by whitefly in a persistent, circulative, non-propagative manner and infect dicotyledonous plant. Bean golden mosaic virus (BGMV) is of this type.

The geminivirus genome is organised in one (monopartite) or two (bipartite) covalently closed, circular, ssDNA molecules of about 2.5-2.9 Kb (Lazarowitz, 1992).

The genes in monopartite and bipartite geminiviruses are arranged in two divergent cluster 280 to 350 nucleotide each separated by the intergenic region (IR) each. The single genomic components of monopartite geminiviruses contain all the information necessary for virus replication and infectivity (Lazarowitz, 1992).

2.5.2 Historical background

Tomato leaf curl virus (ToLCV) disease was first time reported in Israel in 1939-1940 associated with outbreaks of *Bemisia tabaci*. Twenty years later, in 1959, the entire tomato crop was destroyed by a disease with TYLCV-like symptoms in the Jordan Valley. Cohen and Harpaz (1964) published the first description of the new disease transmitted by the whitefly *Bemisia tabaci*.

Before virus isolation, the detection and diagnosis of TYLCV relied on symptom expression, transmission mode and host range. This situation led to

some confusion, since the variety of symptoms associated with TYLCV disease makes it difficult to identify. In this wisdom, tomato leaf curl disease caused by the Tomato leaf curl virus (ToLCV), reported from Sudan, India and Australia has been considered caused by the same viral agent, TYLCV. However, more studies consider both diseases caused by different viral agents (Dry *et al*., 1993).

2.5.3 Symptomology

In tomato ToLCV symptoms vary depending on the growth stage. If disease infection occurs at an early stage of the plant development some changes is happen such leaf size, deformation of leaflets, upward cupping, puckering of leaflets, chlorosis of leaf margin, mottling, flower abscission and partial or complete sterility in the tomato (Sastry and Singh, 1973).

Yassin and Nour (1965) described tomato leaf curl symptoms *viz*., leaf curling, stunting of the plant growth, thickening, greening of the veins of the leaves as similar to those described by Vasudeva and Sam Raj (1948).

The growth and development of tomato plants infected by leaf curl virus were considerably delayed. The disease was also changing by decreased content of green and yellow pigments, increased total nitrogen and accumulation of hexose and sucrose.

2.5.4 Host range of ToLCV

In nature, the virus mainly infects tomato. The experimental host range of ToLCV is narrow, mainly infecting some species of the Solanaceae, Composite and Caprifoliaceae. Vasudeva and Sam Raj (1948) reported that ToLCV exhibits leaf curl symptoms on *Nicotiana tabaccum* L. cvs. White Burley, Samsum and Harrison special, *Solanum tuberosum* L. cv. Craig defiance, *Datura stramonium* L., *N. Sylvestris spegaz* and *N. glutinosa* L. when inoculated by grafting.

Sastry *et al*., (1978) listed three different categories viz., weeds, ornamental and cultivated plants as host plants which had been harbouring

ToLCV as well as vector, *B. tabaci*. Out of the 32 different plant species listed, some of them acted as reservoir not only for virus but also for the whitefly throughout year.

Saiga and Muniyappa (1989) reported that tomato plants were susceptible to infection by ToLCV at all stages of their growth. The incidence of ToLCV In some tomato growing areas of Karnataka, India, ranged from 17-53 per cent in July-November to 100 per cent in crop grown in February-May (summer).

2.5.5 Virus transmission

The virus is transmitted in nature by *B. tabaci* in a semi persistent manner. Maximum acquisition and inoculation feeding periods are 15-30 minutes. The latent period in the vector is more than 20 hours. The virus is retained by the vector for up to 20 days but not throughout the life span insect, but is not transmitted to the progeny. Whiteflies can carry a finite number of virions, in the range of 600 million, indicating that their acquisition. A single whitefly is able to transmit the virus and the rate of transmission increases with population density of the vector (Mansour and Al Musa, 1992).

Although symptoms usually appear at about 15 day's post-whitefly inoculation, viral DNA can be detected 7 days earlier. Young leaves and apices are best for inoculation by whiteflies (Ber *et al*., 1990).

Mechanical transmission has not been possible and there are no reported cases of transmission through seed.

2.6 Heterosis of tomato

Determinate types with negative heterosis or no heterosis would be appreciated. Heterosis for plant height in tomato was reported by Naidu (1993); Bhushana, (2000) and Patil, (2001).

The number of fruits per plant is considered as an important component of fruit yield. Manifestation of heterosis for fruit per plant has been reported by Dixit *et al*. (1980), Prabhushankar (1990), Bhushana (2000) and Patil (2001).

Dixit *et al*., (1980), Prabhushankar (1990) and Bhushana (2000) have been reported significant positive heterosis for this trait, while Patil (2001) reports significant positive and negative heterosis.

A wide range is observed for midparental, better parental and standard heterosis. Dixit *et al*. (1980), Prabhushankar (1990), Bhushana (2000) and Patil (2001) reported significant positive heterosis.

2.7 Conventional breeding for bacterial wilt and ToLCV in tomato

Tomato is highly susceptible to diseases, pests, nematodes and certain environmental factor. Therefore, development of resistant varieties is great significance in tomato production. Since there are a large number of diseases and insect pests, resistant cultivars to more than one disease or insect pests would have a wider acceptance.

In wild species have large number of resistance genes, may be for disease, insect pests or adverse environmental factors, but it is difficult to combine these genes from the wild species to commercial cultivars due to the problem of interspecific hybridisation and linkage of undesirable characters with resistance gene. Considering these problems, it is convenient to use the source of resistance from the cultivated varieties.

2.7.1 Resistance breeding for diseases

2.7.1.1 Bacterial wilt

A stepwise approach to genetic improvement was adopted to rationally address the complexities of factors affecting the tropical adaptation of tomato (Opena, 1985). In the early years, the breeding for heat tolerance and BW resistance was emphasized because these features were considered fundamental to

successful tomato production in the tropics (Opena *et al.,* 1987; Villareal and Lai, 1979).

The bacterial wilt resistant gene was introduced into commercial tomato cultivars from wild species *S. peruvianum*, using embryo rescue of an interspecific cross of the wild species with *S. lycopersicum* (Smith, 1996).

The field trials carried out at North Carolina in USA, cultivars Louisiana Pink and T-414 from Puerto Rico shoed good resistance to bacterial wilt (Schaub and Baver, 1944). Similarly trials conducted resistance in Sri Lanka involving several North Carolina lines indicates resistance in Masterglobe and Rahangala to bacterial wilt (Abeygunawardena and Siriwardene, 1963).

There are also indications that fairly simple genetic control may underlie the BW resistance in some resistant stocks originating from the tropical areas, e.g. the Philippines, as noted by Indian tomato breeders (Tikoo *et al.*, 1990). In another study, it was postulated that a two-gene model with epistasis adequately explained the observed segregation of the hybrid progenies of BW2 line from North Carolina (Bosch *et al.,* 1985).

The tropical tomato has been further improved by sequentially adding new disease resistances such as resistance to tomato mosaic virus (mainly gene *T*) and to rootknot nematode (gene *Mi* for resistance to *Meloidogyne incognita*); and by improving fruit quality including resistance to fruit cracking, improved fruit firmness and size (AVRDC, 2000; AVRDC, 2001).

The genetics of resistance are complex and controlled by polygenes. The resistance genes identification in *S. pimpinellifolium* is partially dominant at seedling stage (Acosta, 1972) whereas in Lousiana pink these factors are recessive (Singh, 1961). Resistance sometimes shift from season and this phenomenon is associated with polygenes (Nelson, 1974). Resistance is associated with poor fruit quality particularly small size which continues to persist in subsequent generations (Acosta *et al*., 1964).

Nelson (1974) while working on tomato concluded that the level of bacterial wilt resistance varied from season to season and was associated with polygenes. Tikoo (1987) reported genotype dependent gene action for resistance to bacterial wilt in tomato. IHR 663-12-3 proved to have a single dominant gene for resistance, but a few other genotypes were observed to have recessive genes.

Regarding the biochemical basis of resistance the resistant plant have higher concentration of steroidal glycoalkaloid (α-tomatine) before and after inoculation than the susceptible plant. The resistant varieties have higher phenol content than the susceptible (Kao and Hsiao, 1980).

In an experiment conducted by Henderson and Jenkins (1972) to evaluate resistance in several genotypes, they found the genotypes such as Venus, Saturn and Beltsville-3814 to be resistant to bacterial wilt.

The resistance in Hawaii 7998 can be traced to PI 127805A, a *S. pimpinellifolium* line (Gilbert *et al*., 1973). Mew and Ho (1976) found that the line VC-8-1-2-1 was resistant to *P. solanacearum* irrespective of the inoculum density.

Sonoda *et al*. (1979) have been identified several sources of resistance to Florida isolates of the pathogen. The best sources of resistance among them are H7997, CRA 66 and PI 126408.

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-0-19 GS cultivars. Similarly Celine (1981) reported field tolerance in tomato in the line CL 32 d-0-1019 GS.

Nirmala Devi (1987) reported that resistance to bacterial wilt in CRA 66 sel A under polygenic control.

Sadhankumar (1995) screened 68 tomato genotypes for resistance to bacterial wilt and found that Sakthi, LE-79-5, LE-415, LE-214, CAV-5 and LE-

382-1 were resistant and said that the genes responsible for resistance in these lines were recessive.

Several tomato varieties such as, Arka Abhijit, Arka Abha, Arka Alok, Sakthi, Mukthi, Anagha resistant to bacterial wilt have been developed through conventional breeding. (Yadav, 2011).

Bacterial wilt resistant varieties/hybrids released by different institutes (Jyothi *et al*., 2012)**:**

1. IIHR: Arka Abha, Arka Abhijit, Arka Alok, Arka Shreshta, Arka Ananya (ToLCV + BW), Arka Rakshak (ToLCV + BW + Early blight), Arka Samrat (ToLCV + BW + Early blight).

2. UAS, Dharwad: Megha.

3. KAU: Anagha, Sakthi, Mukthi and Vellayani Vijay.

4. Orissa University of Agriculture and Technology: Utkal Pallavi (BT-1), Utkal Kumari (BT-10), Utkal Deepti (BT-2) etc.

2.7.1.2 Tomato leaf curl virus (ToLCV)

Despite the absence of immune cultivars observed for majority of the commercial crops affected by ToLCV disease by *B. tabaci*-transmitted geminiviruses, breeding for disease resistance has proven the most complementary and sustainable of the integrated whitefly/begmovirus control methods.

The situation in the Old World is different due to the severe damage caused by a group of begomoviruses collectively referred to as Tomato yellow leaf curl virus (TYLCV) in the Mediterranean region, the Middle East, North Africa, Central Africa and Southeast Asia (Czosnek and Laterrot, 1997). One of these TYLCV variants was accidentally introduced in the last decade into the

America (Nakhla *et al*., 1994; Polston *et al*., 1994), where it has already caused millions of dollar worth of industrial and fresh tomato production losses.

2.7.1.3 Sources of tomato leaf curl virus (ToLCV) resistance

An effective screening procedure, at large scale makes the ToLCV breeding programme more efficient. Many successful screening programmes for ToLCV resistance were carried out in the field. Now days molecular breeding is help do develop ToLCV resistance varieties in short duration than conventional breeding.

Hayati (1978) suggested that individual plant inoculation in which white flies were reared on immune eggplant inoculation in which were starved for one hour before transferring to ToLCV infected tomato plants. After 48 hours of acquisition feeding, they were gently removed and allowed to feed on healthy tomato plants to be tested for their resistance to ToLCV at three to four true leaf stages.

Vasudeva and Sam Raj (1948) screened more than sixty varieties of tomato and reported all of them to be susceptible to ToLCV.

Mayee *et al*. (1974) reported that HS-110, HS-102, Nematex, T-1 and Nova are some of the ToLCV tolerant varieties developed by conventional breeding methods.

Hassan *et al*. (1985) tested 46 tomato cultivars and breeding lines against tomato yellow leaf curl virus and found none of them resistant.

Banerjee and Kalloo, (1989) screened 122 varieties, lines and wild accession of *Lycopersicon* and recorded that *S. hirsutum S. typicum*, S. *peruvianum* possessed resistance to ToLCV and observed no disease symptoms in *S. pimpinellifolium* till 90 days of age.

Among *S. lycopersicum* accession LE-812, LE-376 and AVRDC lines were carrying field resistance to ToLCV (Shoba and Armugam, 1991).

Moustafa and Hassan (1993) screened 17 true breeding cultivars, four tolerant hybrids and the total control Castle rock for ToLCV resistance. The four hybrids Typhoon, TY 20, BB 234 and BB 235 and true breeding cultivar T 22 showed better virus resistance.

Two ToLCV resistances varieties (Hisar Anmol and Hisar Gaurav) derived from a backcross pedigree of *S. hirsutum, F. plabratum* X *S. lycopersicum* have been identified by the variety evaluation committee of Haryana Agriculture University, Hissar. The performance of H-24 with respect to yield and reaction to ToLCV under field and artificial inoculation. They found that mean PDI values of H-24, Sel-7 and Punjab Chhuhara were 18.83 %, 50.23 % and 673.57 % respectively.

Sadashiva *et al*. (2003) screened the advanced breeding lines for ToLCV disease resistance along with the susceptible variety Arka Saurabh. The results indicated that the breeding lines TLBR-1, TLBR-2, TLBR-3, TLBR-4, TLBR- 5, TLBR-6, IIHR-2196, IIHR-2197, IIHR-2198, IIHR-2199 and IIHR-2100 had greater tomato leaf curl virus resistance.

A total of 25 lines were screened for tolerance to high temperature and ToLCV. Of which 16 lines *viz*; IIHR-2195, IIHR-2196, IIHR-2197, IIHR-2199, IIHR-2000, IIHR-2201, IIHR-2202, IIHR-2223, IIHR-2230, IIHR-2231, IIHR-2234, IIHR-2239, IIHR-2243, IIHR-48, IIHR-2249 and IIHR-2251 were found to be tolerant to high temperature and resistant to ToLCV and all the lines had high per cent fruit set under field conditions (Singh and Sadashiva, 2007).

Muniyappa and co-workers (1991) reported that lines of S. *hirsutum* and S. *peruvianum* were resistant to another tomato geminivirus, Tomato leaf curl virus (ToLCV). The resistance mechanism in these wild species was associated

with the production of exudates from trichomes glands on the leaf surface, in which whiteflies became entrapped (Channarayappa and Shivashankar, 1992).

IIHR 2196 resistant to ToLCV have been developed through conventional breeding (Yadav, 2011).

2.8 Marker assisted selection in crops

Molecular markers are by-products of genomic research and investigation, and as aids to selection, fit within the genome-wide concept of observed functional relationship between the genome and phenotype. Linkage imbalances between distinguishable molecular marker allelomorphs and genes controlling the development of a phenotypic trait can be exploited to establish an empirical correlation between distinct allelomorphs and of the molecular markers with level of phenotypic expression of the trait. The commonly used molecular markers include; restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), Sequenced characterised amplified regions (SCARs) and single nucleotide polymorphism (SNP), with SNPs currently gaining in favour.

The essential requirements for marker-assisted selection in a plant breeding programme are: markers should co-segregate or be closely linked (1 cM or less is probably sufficient for MAS) with the desired trait; an efficient means of screening large populations for the molecular markers should be available. At present, this means, relatively easy analysis based on PCR technology; the screening technique should have high reproducibility across laboratories, be economical to use and be user-friendly.

Markers can be used for the breeding of crops and they accelerate conventional breeding programme. Screening by molecular markers (linked to specific genes) is quick and accurate.

2.8.1 Polymerase chain reaction (PCR)

The Polymerase Chain Reaction (PCR) technique has been the basis of a growing range of new techniques for genome analysis based on the selective amplification of genomic DNA fragments (Saiki *et al*., 1988). The PCR reaction requires deoxynucleotides, DNA polymerase, primer, template and buffer containing magnesium (Taylor, 1991). Typical PCR amplification utilises oligonucleotide primers which hybridise to complementary strands. The product of DNA synthesis of one primer serves as template for another primer. The PCR process requires repeated cycles of DNA denaturation, annealing and extension with DNA polymerase enzyme, leading to amplification of the target sequence. This results in an exponential increase in the number of copies of the region amplified by the primer (Saiki *et al*., 1988). The technique can be applied to detect polymorphism in various plants, animals, bacterial species and fungi.

2.8.2 PCR- based molecular marker techniques

DNA fingerprinting is a technique, which has been widely adapted to differentiate organisms at the species and subspecies levels (McClean *et al*., 1994). The techniques used for cultivar identification are designed to detect the presence of specific DNA sequences or combination of sequences that uniquely identify the plant. Cultivar identification can be achieved more accurately using DNA fingerprinting data, especially in materials characterized by high genetic variations between cultivars. The most closely related cultivars are usually distinguished with the DNA fingerprinting methods. The application of DNA fingerprinting could be very valuable in the identification of cultivars and species and could help to create more efficient breeding programs through the detection of genetic linkages between DNA fingerprinting bands and agriculturally important quantitative trait loci (QTL). The high variability of DNA fingerprinting described in humans, animals and plants allows the identification of different individuals, genotypes, and species (Lin *et al*., 1993). It is help to develop specific marker which is close to specific gene of interest.

2.8.3 Molecular markers in different crops

In breeding for disease and pest resistance, at present, the segregating populations derived from crosses between the resistant sources. Pathogens and insects are known to overcome resistance provided by single genes. Durability of resistance has been increased in several crops by incorporating genetic diversity of the major resistance genes. MAS for resistance genes (R) can be useful in all these approaches. With MAS, new R gene segregation can be followed even in the presence of the existing R gene(s) and hence R genes from diverse sources can be incorporated in a single genotype for durable resistance. Pyramiding of bacterial blight resistance genes *Xa1*, *Xa3*, *Xa4*, *xa5*, and *Xa10* in different combinations using molecular markers has recently been reported (Yoshimura *et al*., 1995).

Cao *et al*., (2009) reported that the inheritance of bacterial wilt resistance in eggplant was controlled by a single dominant gene showing Mendelian inheritance model. In addition, a 762 bp molecular marker linked to a bacterial wilt-resistant gene of eggplant was screened by the bulked segregant analysis (BSA) method and sequence characterized amplified region (SCAR) marker linked to bacterial wilt-resistance gene was also obtained. The genetic distance between this marker and the resistance gene is 3.33 cM.

2.8.4 Molecular markers in tomato

Tomato was one of the first crops for which molecular markers were suggested as indirect selection criteria for breeding (Tanksley and Hewitt, 1988). The actual use of MAS in tomato breeding began approximately 30 years ago with the use of the isozyme marker acid phosphatase (*Aps-11* locus) as an indirect selection criterion for breeding for nematode resistance (Filho and Stevens, 1980).

As for quantitative traits, in addition to the limited use of MAS for manipulating QTLs for traits such as fruit flavour and soluble solids content (◦Brix), MAS is being attempted for improving quantitative resistance to diseases

such as powdery mildew, bacterial canker and bacterial wilt. Furthermore, despite considerable efforts devoted to the identification and mapping of QTLs for various abiotic stress tolerance traits in tomato, including salt tolerance, drought tolerance and cold tolerance, it does not seem MAS has been employed for improving any of these traits.

Many QTLs reported for complex traits in tomato are either unreliable, population specific or not strong enough in terms of linkage to warrant their use for marker-assisted breeding. In fact, in many cases where MAS has been employed to transfer QTLs from wild species, there have been problems associated with linkage drag and recovery of desirable horticultural characteristics (Brouwer and St. Clair, 2004; Collard *et al.*, 2005). Such undesirable associations could be due to genetic linkage and/or pleiotropic effect, the distinction between the two is often not very straightforward.

2.8.4.1 Molecular markers for bacterial wilt resistance in tomato

Resistance genes or QTLs and molecular markers associated with resistance have been identified in tomato for the various bacterial diseases, including bacterial canker, bacterial speck, bacterial spot and bacterial wilt. It has been hoped that molecular markers and MAS would facilitate breeding for resistance to bacterial diseases in tomato, however, various constraints, including limitations in the availability of resistance-linked markers and insufficient marker polymorphism (Yang and Francis, 2007), have restricted the progress.

For instance, the mapping populations derived from a cross between West Virginia 700 and Hawaii 7996 have been used to map quantitative trait loci (QTLs) associated with bacterial wilt and late blight, but the low number of polymorphic markers between these two parents has been an obstacle towards fine-mapping and marker assisted selection (MAS) of these QTLs (Thoquet *et al*., 1996; Moreau *et al*., 1998; Wang *et al*., 2000).

Table 1.Selected examples of association of molecular markers with the desired traits in different crops.

Characters	Examples	References
Rice		
Resistance to blast caused by *Pyriculariaoryzae*	*Pi-2(t)* gene located 2.8 cM from RG64 on chromosome 6 *Pi*-4(t) gene located15.3 cM from RG 869 on chromosome 12	Yu *et al*. (1991)
	Pi-10t gene tagged with RAPD markers RRF6 and RRH18 on chromosome 5	Naqvi *et al*. (1995)
Resistance gene *Xa21* to bacterial blight caused by *Xanthomonas oryzae*	Located 5.3 cM away from RAPD marker	Zhang *et al*. (1994)
	RAPD818, RAPD248 and RG103 co-segregated	Ronald *et al*. (1992)
Xanthomonas oryzae	*xa5* with RFLP markers RZ390, RG556, RG207 on chromosome 5;*Xa3* and *Xa4* with RFLP locus XNpb 181 and *Xa10* with RAPD marker O072000 on chromosome 11	Yoshimura *et al*. (1995)
Gall midge resistance	1.3 cM from marker	
Gm2 gene	RG329, 3.4 cM from marker RG476 on chromosome 4	Mohan *et al*. (1994)
	RAPD fragment F81700 associated	Nair *et al*.

Gm2 gene	with susceptibility and F10600 with resistance	(1995)
Gm4t gene	RAPD fragment E20570 associated with resistance and E20583 with susceptibility	Nair *et al.* (1996)
Wheat		
*Triticumtauschii*cereal cyst nematode resistance *Ccn-D1* gene	Complete cosegregation with RAPD fragment csE20-2	Eastwood *et al.* (1994)
Leaf rust resistance *Lr24* gene	6 RFLP markers completely linked	Schachermayr *et al*. (1995)
Lr9 gene	Complete linkage of 3 RAPD markers and one RFLP marker	Schachermayr *et al*. (1994)
Powdery mildew resistance Powdery mildew resistance	RFLP markers 3 cM from genes *Pm-1* and *Pm-2 Pm*-1 3 cM from *Pm*-2	Hartl *et al*. (1995)
Tomato		
Insect resistance mediated by 2-tridecanone (2-TD)	Direct selection for RFLP loci increased the frequency of 2-TD-mediated resistance	Nienhuis *et al*. (1987)
MV resistance *Tm*-2 locus	4 cM from introgressed region	

Resistance to *Fusarium oxysporum*: *I2* gene introgressed from *S pimpinellifolium*	RFLP marker linked to *I2*	Young and Tanksley (1989)
Il gene introgressed from S. *pennellii*	RFLP markers TG20 and TG18 on chromosome 7	Sarfatti *et al*. (1991)
Nematode resistance *Mi* gene	RFLP markers tightly linked	Klein-Lankhorst *et al*. (1991)
Cladosporium fulvum resistance	Close linkage between *Cf*-2, *Cf-5* and *Mi*	Dickinson *et al*. (1993)
Resistance to powdery mildew caused by *Oidium lycopersicum O1-1 gene*	Near *Aps-1* region on chromosome 6 close to *Mi and Cf-2/Cf-5 genes*	Jacob *et al*. (1991)
Soluble solid content (SS)	*RFLPs linked to SS*	Osborn *et al*. (1987)
Potato		
Race-specific resistance to *Phytophthora infestans R1 allele*	Flanking RFLP loci-GP21 and GP179 and 2 AFLP loci not separated from *R1 by recombination*	MeKsem *et al*. (1995)

Simple sequence repeat (SSR) markers are often the preferred molecular markers for the purpose of marker-assisted plant breeding when they are available, because the SSR markers possess properties suitable for high-throughput genotyping, such as high reproducibility, co-dominance nature, multi-allelic variation, simplistic assay, low distributing cost and easy automation (Edwards and McCouch, 2007).

The conventional method for SSR marker development involves construction of genomic libraries and screening them for repeat motifs (Zane *et al*., 2002). Areshchenkova and Ganal (2002) used this approach to develop 32 SSR markers for tomato with longer repeats. However, this approach is cumbersome and intensive in terms of cost, time and labour. Alternatively, DNA sequences deposited in public databases provide an easy and economical source for development of SSR markers (Morgante and Olivieri, 1993).

The tomato genome sequencing project has generated sequences of many bacterial artificial chromosome (BAC) clones which augments the existing genomic resources (Mueller *et al*., 2009). The search for repeat motifs in genomic sequences and expressed sequence tags (ESTs) of Solanaceae family available in the European Molecular Biology Laboratory (EMBL), GenBank and Solanaceae Genomics Network (SGN) databases enabled to rapidly produce a few hundred SSR markers in tomato (Smulders *et al*., 1997; He *et al*., 2003; Frary *et al*., 2005).

Currently, a total of 404 tomato SSR markers have been developed and mapped. Information for 256 and 148 SSR markers is available at SGN and Vegmarks, respectively (http://www.sgn.cornell.edu). Nevertheless, these SSR markers are not distributed evenly throughout the 12 chromosomes, and tend to cluster around the centromeres (Areshchenkova and Ganal, 2002; Ohyama *et al*., 2009). This phenomenon may be attributed to the fact that a large proportion of SSR markers developed from BAC end sequences were located predominantly in the heterochromatin regions. Most of these SSR markers have not been evaluated for their ability to detect genetic variation within cultivated and closely related

tomato species. The number of polymorphic markers is expected to be less for closely related cultivated tomatoes.

In most cases resistance has been reported to be polygenic (Danesh *et al.*, 1994; Thoquet *et al.*, 1996; Hanson *et al.*, 1998; Mangin *et al.*, 1999), although in a few cases the presence of major resistance genes has been suggested. In particular, a single dominant resistance gene was reported in Hawaii 7998 (Scott *et al.*, 1988) and Hawaii 7996 (Grimault *et al.*, 1995). Traditional breeding for bacterial wilt resistance has proven difficult for various reasons, including variation in pathogen populations, uncontrollable environmental effects on disease expression, and association of resistance with undesirable horticultural characteristics such as small fruit size (Scott *et al.*, 2005; Yang and Francis, 2007).

Using different resistance sources and pathogen strains, several QTLs for resistance to bacterial wilt were identified on tomato chromosomes 3, 4, 6, 7, 8, 10 and 12 (Thoquet *et al.*, 1996; Mangin *et al.*, 1999). Among these, one genomic location with large effect on chromosome 6 (consisting of one or more QTLs) has been repeatedly identified (Mangin *et al.*, 1999; Wang *et al.*, 2000; Miao *et al.*, 2009; Geethanjali *et al.*, 2010). In a recent study, molecular markers associated with bacterial wilt resistance in a highly-resistant cultivar (T51A) were reported (Miao *et al.*, 2009). In this cultivar, the bacterial wilt resistance was controlled by two complementary, co-dominant genes located on tomato chromosome 6. Two dominant SCAR markers, $TSCAR_{AAT/CGA}$ and $TSCAR_{AAG/CAT}$, were reported to be located 4.6 cM and 8.4 cM, respectively, from a resistance gene, *TRSR-1*, and have been suggested useful for breeding for bacterial wilt resistance via MAS (Panthee and Foolad, 2012).

Moreover, a major QTL associated with bacterial wilt caused by *Ralstonia solanacearum* in Hawaii 7996 has been mapped on chromosome 6 (Thoquet *et al*., 1996; Wang *et al*., 2000; Carmeille *et al*., 2006). And several genes and QTLs conferring resistance to various biotic stresses such as bacterial canker (Sandbrink

et al., 1995; Van Heusden *et al*., 1999), powdery mildew (Bai *et al*., 2003), leaf mold (Jones *et al*., 1993), tomato yellow leaf curl virus (Zamir *et al*., 1994; Hanson *et al*., 2000) and root knot nematode (Ammiraju *et al*., 2003) were mapped to chromosome 6. Additional useful SSR markers on chromosome 6 would facilitate the transfer of these useful alleles in breeding processes.

Mangin *et al*. (1999) was analysed the data from a field test F_3 population, show that at least two separate loci ~30 cM apart on this chromosome are most likely involved in the resistance. First, a temporal analysis of the progression of symptoms reveals a distal locus early in the development of the disease. As the disease progresses, the maximum LOD peak observed shifts toward the proximal end of the chromosome, obscuring the distal locus. These results are discussed in the context of current molecular knowledge about disease resistance genes on chromosome 6and observations made by tomato breeders during the production of bacterial wilt-resistant varieties.

Siri *et al*. (2009) studied 30 *S. commersonii* clonal accessions, 20 of which are resistance against *R. solanacearum* was tested and diVerent levels of resistance were found, ranging from delayed wilting to asymptomatic reactions. The genetic variation and the relationships among individuals in this germplasm collection were studied by diVerent molecular markers: Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Microsatellites or Simple Sequence Repeats (SSR). AFLP markers generated the largest number of total and polymorphic fragments per assay unit while SSR revealed the highest frequency of polymorphic bands (100%), followed by AFLP (96.2%) and RAPD (89.4%). In contrast, when comparing the number of diVerent genetic proWles generated, the SSR markers exhibited the lowest discriminatory power. The clustering pattern obtained with the three marker systems showed a similar distribution of the *S. commersoniis* germplasm revealing a high correlation between the three methods employed.

The SOL Genomics Network (http://sgn.cornell.edu) is a rapidly evolving comparative resource for the plants of the Solanaceae family, which includes important crop and model plants such as potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), pepper (*Capsicum annuum*), and tomato (*Solanum lycopersicum*). The aim of SGN is to relate these species to one another using a comparative genomics approach and to tie them to the other dicots through the fully sequenced genome of Arabidopsis (*Arabidopsis thaliana*). SGN currently houses map and marker data for Solanaceae species, a large expressed sequence tag collection with computationally derived unigene sets, an extensive database of phenotypic information for a mutagenized tomato population, and associated tools such as real-time quantitative trait loci (Mueller *et al*., 2005).

2.8.4.2 Molecular markers for tomato leaf curl virus resistance in tomato

Genes or QTLs and resistance-linked markers have been identified and reported for several viral diseases of tomato, including cucumber mosaic virus, potyviruses, tomato mosaic virus, tomato mottle virus, tomato spotted wilt virus, tomato yellow leaf curl virus and tomato leaf curl virus. Here we describe the status of marker development and their use for breeding tomatoes with resistance to tomato leaf curl virus.

Scott (2007) reported that the disease response of the resistant cultivars often varies from location to location, and it has been difficult to develop resistant cultivars with horticultural characteristics similar to those of susceptible ones. Four resistance loci, *Ty-1, Ty-2, Ty-3* and *Ty-4*, have been identified and mapped to tomato chromosomes 6, 11, 6 and 3, respectively (Zamir *et al.*, 1994; Chague *et al.*, 1997; Ji *et al.*, 2009a; Ji *et al.*, 2009b). Several QTLs conferring resistance to TYLCV have also been identified (Agrama and Scott, 2006). At least six PCR-based molecular markers associated with the major resistance genes have been developed and reported (Foolad and Panthee, 2012).

Yuanfu *et al*., (2009) has been introgressed to cultivated tomato (*Solanum lycopersicum*) from *Solanum chilense* accessions LA1932 and LA2779. A major

gene, *Ty-3*, responsible for resistance to ToMoV and TYLCV was previously mapped on the long arm of chromosome 6. In the present study, we identified a 14-cM *S. chilense* introgression on the long arm of chromosome 3 in some resistant breeding lines derived from LA1932. A new begomovirus resistance locus, *Ty-4*, was mapped to the 2.3-cM marker interval between C2_At4g17300 andC2_At5g60160 in the introgression. Analysis of a population segregating for *Ty-3* and *Ty-4* demonstrated that *Ty-3* accounted for 59.6% of the variance, while *Ty-4* only accounted for 15.7%, suggesting that *Ty-4* confers a lesser effect on TYLCV resistance. Recombinant inbred lines (RILs) with *Ty-3* and *Ty-4* had the highest level of TYLCV resistance. The PCR based markers tightly linked to the *Ty-4* locus as well as the *Ty-3* locus have been recently used in our breeding program for efficient selection of high-levels of begomovirus resistance and now allow for efficient breeding by marker-assisted selection.

Nazeem *et al*., (2010) screened 139 random decamers analysis, 16 markers were identified. Few of the primers were amplified with polymorphic bands in some of the LE verities (LE-12, LE-66 and LE-20) such as OPHA 12, OPF 14, OPBG 7, OPF 9, OPN 6, OPN 19, OPS 1, OPS 7, OPS 15, OPS 16, OPS 17, OPS 20and OPY 10. The number of amplicons produced by different primers ranged from 3 to 12 and the product size ranged from 0.75 Kb to 3.5 Kb. Two putative QTL were found on chromosome 3 and 8. In addition, a weak putative QTL was detected on chromosome 10. The RAPD a marker with the primer of OPHA 12 was converted to a SCAR marker, for its convenient use in marker assisted selection for bacterial wilt and ToLCV resistance in tomato. However, the SCAR marker developed and analysed was not efficient to discriminate resistant and susceptible genotype in tomato both in the case of bacterial wilt and ToLCV.

Nazeem *et al*., (2010) analysed 107 SSR primers out of them 28 markers were identified as polymorphic and 25 were found to have trait specificity. About 12 markers were identified for ToLCV resistance and 8 markers identified for bacterial wilt resistance. Primer SSR 450, SSR 306, SSR 285, SSR 310, SSRKAU 11, LEaat002, LEaat007, LEaat014, and LEaat008 produced distinct

polymorphism for ToLCV and SSR 450, SSR 306, SSR LEaat001 and LEaat 015 produced distinct polymorphism for bacterial wilt incidence in tomato.

Nazeem *et al*., (2010) analysed 21 ISSR primers for distinct polymorphism, out of them ISSRKAU 9, ISSRKAU7 and ISSRKAU 4 which is developed from SSR markers shown the trait specific polymorphism.

Gonza´lez-Cabezuelo *et al*., (2012) reported several genes conferring resistance to TYLCV, mainly *Ty-1* and *Ty-3* genes have been introgressed to cultivated tomato (*Solanum lycopersicum*) from the wild relative species *Solanum chilense*. By combining bulked segregant analysis and amplified fragment length polymorphisms (AFLP), several AFLP markers closely linked to *Ty-1* and *Ty-3* genes were identified from the resistant breeding line TZ841-4.

Cloning and sequencing of the selected AFLP fragments allowed us to develop co-dominant cleaved amplified polymorphic sequence and dominant sequence characterized amplified region markers closely linked to *Ty-1*. In addition, *Ty-3*-linked allelic specific markers have been discriminated by a quantitative real-time PCR protocol. Taken together, these markers constitute useful tools for marker-assisted selection breeding programs to improve genetic resistance to TYLCV, and also to initiate map-based cloning approaches to isolate the resistance genes (Gonza´lez-Cabezuelo *et al*., 2012).

Materials and Methods

3. MATERIALS AND METHODS

The integration of ToLCV resistance in bacterial wilt resistance background was carried out at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agriculture University, Vellanikkara, Thrissur. The methodology and materials used in this experiment are described in this chapter.

3.1 Materials

3.1.1 Plant materials

3.1.1.1 Parent plant materials

Bacterial wilt resistant variety Sakthi, released from Kerala Agricultural University and ToLCV resistant genotype IIHR 2196 from Indian Institute of Horticulture Research were used for developing the combined disease resistance. These genotypes were grown under net house conditions in sterile growth media during January-April, 2013 for extraction of DNA samples (Plate 1). Thirty days old seedlings were transplanted in pots filled with formaldehyde sterilised potting mixture. The cultural and agronomic practices were followed as per the package of practices recommended by KAU, 2011.

3.1.1.2 Source of F_2 seeds

F_2 population derived from a cross between the bacterial wilt resistant genotype Sakthi and ToLCV resistant genotype IIHR 2196 have been developed as part of imparting combined resistance in tomato in the Sol Genome Programme implemented at KAU. Seeds were collected from selected F_2 plants with combined resistance to bacterial wilt and ToLCV. These included plants F_2-06, F_2-18, F_2-20 and F_2-33. The seeds from these plants were used for raising the F_3 population (Plate 2).

a. Sakthi (Bacterial wilt resistant) and fruit shape (Circular)

b. IIHR 2196 (ToLCV resistant) and Fruit shape (Cylindrical)

Plate 1: Parent genotypes used in the study and their fruit shapes

Plate 2: Screening of F_3 population for bacterial wilt and ToLCV in the field

3.1.2 Laboratory chemicals, glasswares and equipment items

The chemicals used in the study were of good quality (AR grade) procured from Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The *Taq* DNA polymerase, dNTPs, Taq buffer and molecular markers used were supplied by Bangalore Genei Ltd. or Sigma. All the plastic ware used were obtained from Axygen and Tarson India Ltd. ISSR, SSR and SCAR primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd. Centrifugation was done in High speed refrigerated centrifuge (KUBOTA 6500/Applied Biosystem). NanoDrop^{R} ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. The DNA amplification was carried out in Veriti Thermal Cycler (Applied Biosystem) and Agilent. Agarose gel electrophoresis was performed in horizontal gel electrophoresis unit (BIO-RAD, USA).

3.2 Methods

3.2.1 Morphological characteristics and disease reactions

Morphological characters of tomato varieties/accessions were observed (Sakthi and IIHR 2196) and F_3 was raised in disease sick field for disease screening for bacterial wilt and ToLCV at Department of Olericulture, College of Horticulture. The morphological parameters were recorded as per the minimal descriptor of NBPGR. The characters observed were as follows:

1. Days to flowering
2. Days to fruiting
3. Branching pattern
4. Plant height (cm)
5. Fruit size and shape
6. Fruit weight (g)
7. Number of fruits per plant
8. Yield per plant (g)
9. Bacterial wilt incidence (%)

10. Disease reaction, severity and ToLCV incidence

11. Number of plant infected

12. Per cent of infection

1. Bacterial wilt incidence

Incidence of bacterial wilt was recorded and when wilt was observed, final count was computed. The F_3 population lines were classified into four groups as suggested by Mew and Ho (1976). The wilt incidence was confirmed by ooze test (Plate. 3). Per cent wilt incidence was calculated by the following formula:

$$\text{Per cent disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Based on the per cent disease incidence the F_3 population was classified into four groups:

PDI (%)	**Disease reaction**
0-20	Resistant
20-40	Moderately Resistant
40-60	Moderately susceptible
60-100	Susceptible

2. Disease reaction, severity and ToLCV incidence

Based on the per cent of curling and puckering of leaves (Plate 4), the plant was scored in 0-4 scale as suggested by Banerjee and Kalloo (1987). 0-4 scale was assigned as detailed below:

0 : Symptoms absent

1 : Very mild curling (Upto 25 % leaves)

2 : Curling and puckering of 26-50% leaves

a. Bacterial wilt infected plant

b. Ooze test for bacterial wilt confirmation

Plate 3: Bacterial wilt confirmation through Ooze test

3 : Curling and puckering of 51-75% leaves

4 : Severe curling and puckering of >75% leaves

Based on the disease score, per cent disease severity (PDS) was calculated using the following formula:

$$\text{PDS} = \frac{\text{Sum of numerical rating}}{\text{Total number of plants observed} \times \text{Maximum disease grade}} \times 100$$

Per cent disease incidence (PDI) was calculated using the following formula.

$$\text{PDI} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Based on the per cent disease severity (PDS) and per cent disease incidence (PDI) the coefficient of the infection (CI) was calculated using following formula.

$$\text{Coefficient of infection} = \frac{\text{PDS} \times \text{PDI}}{100}$$

Based on the coefficient of infection the genotypes were categorised into six groups.

0-4 : Highly resistant (HR)

4.1-9 : Resistant (R)

9.1-19 : Moderately Resistant (MR)

19.1-39 : Moderately susceptible (MS)

39.1-69 : Susceptible (S)

69.1-100 : Highly susceptible (HS)

a. ToLCV infected plant (Early stage)

b. ToLCV infected plant (Fruiting stage)

Plate 4: ToLCV infected plants at early stage and fruiting stage

3. Days to flowering

The number of days from date of transplanting to the appearance of first flower was recorded.

4. Days to fruiting

Number of days taken by a plant to set the first fruit from date of transplanting was recorded.

5. Growth habit

Plants were classified in three groups such determinate, semi-determinate and indeterminate according to branching pattern.

6. Plant height

The plant height was measured from ground level to tip of the plant at the time of crop senescence and plant height expressed in centimetres.

7. Fruit size and shape

Fruit size and shape was recorded for all the survived plants.

8. Fruit weight

The harvested fruits weight were recorded and expressed in gram.

9. Number of fruits per plant

Fruits harvested periodically from each plant were added to obtain the total number of fruits per plant.

10. Yield per plant

The weight of fruits from each picking was recorded from each plant. Total yield per plant was worked out by adding yield of all the harvests fruits and were expressed in gram per plant.

3.2.2 Molecular analysis

Molecular analysis of the parent genotypes and F_3 population were carried out with three different marker systems- Inter simple sequence repeats (ISSR), simple sequence repeats (SSR) and Sequenced characterised amplified region (SCAR).

3.2.2.1 Genomic DNA extraction

Young tender, pale green leaves (first to third from the tip) were collected on ice from each of the 200 plants. The surface was cleaned by washing with sterile water and wiping with 70 per cent alcohol. The fresh leaves were ground into a fine powder in liquid nitrogen along with β -mercaptoethanol and PVP using ice-cold mortar and pestle in order to prevent browning due to phenol oxidase activity.

Among the most commonly used protocols, CTAB method developed by Rogers and Bendich (1994) was used for the extraction of genomic DNA. Details are as follows

Reagents

I. CTAB buffer (2X):

- 2 per cent CTAB (w/v)
- 100mM Tris (pH8)
- 20mM EDTA (pH8)
- 1.4M NaCl
- 1 per cent PVP

II. 10 per cent CTAB solution:

- 10 per cent CTAB (w/v)
- 0.7M NaCl.

III. TE buffer:

- 10mM Tris (pH8)

-1mM EDTA

IV . Chloroform: Isoamyl alcohol (24:1 v/v)

V. Isopropanol

VI. Ethanol 70 per cent and 100 per cent

VII. Sterile distilled water

Procedure:

- 0.2 gram of clean leaf tissues was ground in pre-chilled mortar and pestle in presence of liquid nitrogen.
- 1ml of extraction buffer (2X), 10µl of β-Mercaptoethanol and a pinch of Poly Vinyl Pyrrolidone (PVP) were added to the mortar.
- The homogenized sample was transferred into an autoclaved 2ml centrifuge tube and 1ml of pre-warmed extraction buffer was added (total 1ml).
- The contents were mixed well and incubated at 65ºC for 20 to 30 minutes with occasional mixing by gentle inversion.
- Equal volume (1ml) of chloroform: Isoamyl alcohol (24:1) was added and mixed by inversion to emulsify. Spun at 12,000 rpm for 15 minutes at 4ºC
- After centrifugation the contents got separated into three distinct phases.

Aqueous topmost layer	- DNA and RNA
Interphase	- Fine particles and proteins
Lower layer	- Chloroform, pigments and cell debris

- Transferred the top aqueous layer to a clean centrifuge tube and added 1/10th volume of 10 per cent CTAB solution and equal volume of chloroform: Isoamyl alcohol (24:1) and mixed by inversion.
- Centrifuged at 12,000 rpm for 15 minutes at 4ºC.
- Transferred the aqueous phase into a clean centrifuge tube and added 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till the DNA precipitated. Kept at -20ºC for half an hour for complete precipitation.
- Centrifuged at 10,000 rpm for 05 minutes at 4ºC. Gently poured off the supernatant.
- Washed the DNA pellet with 70 per cent ethanol followed by 100 per cent ethanol.
- Spun for 5 min at 10,000 rpm and decanted the ethanol.
- Air dried the pellet, dissolved in 50µl of sterilized water and stored at -20ºC.
- Loaded the samples on 0.8 per cent agarose gel to observe the quantity and quality of DNA. Composition of reagents is provided in annexure I.

3.2.3 Purification of DNA

The DNA which had RNA contaminant was purified by RNase treatment and further precipitated, washed and air dried.

Reagents,

- Phenol: chloroform mixture (24:1, v/v)
- Chilled isopropanol
- 70 per cent ethanol
- TE buffer
- Chloroform: Isoamyl alcohol (24:1, v/v)

- 1 per cent RNase

One per cent solution was prepared by dissolving RNase (Sigma, USA) in TE buffer at 100^0C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20^0C.

Procedure

- To 100 µl DNA sample, RNase solution (2 µl) was added and incubated at 37^0Cin dry bath for 1 hour.
- The volume was made up to 250 µl with distilled water.
- Added equal volume of chloroform: isoamyl alcohol (24: 1) mixture and mixed gently.
- Centrifuged at 12,000 rpm for 15 minutes at 4^0C.
- Transferred the aqueous phase into a fresh micro centrifuge tube and added equal volume of chloroform: isoamyl alcohol (24: 1)
- Centrifuged at 12,000 rpm for 15 minutes at 4^0C.
- Transferred the aqueous phase into a clean centrifuge tube and added 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till the DNA precipitated. Kept at -20ºC for half an hour for complete precipitation.
- Incubated the mixture at -20^0C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4^0C.
- Washed the DNA pellet with 70 per cent ethanol
- Air dried the pellet, dissolved in 50 to 100 µl sterilized water.
- Loaded the samples on 0.8 per cent agarose gel at constant voltage of 100 V to test the quality and to find whether there was any shearing during RNase treatment.

3.2.4 Electrophoresis of DNA

The quality of isolated DNA was evaluated through agarose gel electrophoresis.

Reagents and equipments,

1. Agarose - 0.8 per cent (for genomic DNA)

   - 1.5 per cent for ISSR and 2 per cent SSR and SCAR samples

2. 50X TAE buffer (pH8.0)

   - Tris buffer

   - Acetic acid

   - 0.5mM EDTA

3. Tracking/loading dye (6X)

   - Bromophenol blue

   - Glycerol

4. Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml) composition of reagents is provided in annexure II.

Procedure

- The gel tray was prepared by sealing the ends with tape. Comb was placed in gel tray about 1 inch from one end of the tray and positioned the comb vertically such that the teeth are about 1 to 2 mm above the surface of the tray.

- Prepared 0.8 per cent agarose (0.8 g in 100ml) in a glass beaker or conical flask with 100 ml 1X TAE buffer. Micro waved for 45 to 60 seconds until agarose was dissolved and solution was clear.

- Solution was allowed to cool to about 42 to 45 ^{0}C before pouring. (4µl Ethidium bromide was added at this point to a concentration of 10 µl/ml) and mixed well.

- Poured this warm gel solution into the tray to a depth of about 5 mm. allowed the gel to solidify for about 30 to 45 minutes at room temperature.

- To run, gently removed the comb and the tape used for sealing, placed the tray in electrophoresis chamber, and covered (just until wells are submerged) with electrophoresis buffer (the same buffer used to prepare the agarose).

- To prepare samples for electrophoresis, added 1 µl of 6x gel loading dye for every 5µl of DNA solution. Mixed well and loaded 6µl DNA sample per well. Loaded suitable molecular weight marker (λDNA *Eco*RI/*Hind*III double digest) in one lane.

- Electrophoresed at 70 volts until dye has migrated two third the length of the gel.

- Intact DNA appears as orange fluorescent bands. If degraded, it appears as a smear because of the presence of a large number of bands, which differ in base length. The gel profile was examined for intactness, clarity of DNA band, presence of RNA and protein.

3.2.5 Gel documentation

Gel documentation was done with BioRad Gel Documentation System using PDQuest™ software. PDQuest is a software package for imaging, analysing, and data basing 2-D electrophoresis gels. PDQuest can acquire images of gels using any of several Bio-Rad imaging systems. An image of a gel is captured using the controls in the imaging device window and displayed on computer screen.

3.2.6 Assessing the quality and quantity of DNA by spectrophotometer

The purity of DNA was further checked by using NanoDrop ND-1000 spectrophometer. Nucleic acid shows absorption maxima at 260nm whereas proteins show peak absorbance at 280nm. Absorbance is recorded at both wavelength and purity is indicated by the ratio OD_{260}/OD_{280}. The values between 1.8 and 2.0 indicate that the DNA is pure and free from proteins. The quantity of DNA in the pure sample was calculated using the relation 1 OD_{260} equivalent to 50 µg double stranded DNA/ml sample.

1 OD at 260 nm = 50 µg DNA/ml

Therefore OD_{260} X 50 gives the quantity of DNA in µg/ml.

Procedure for quantity detection using Nanodrop

- Connected the Nanodrop spectrophotometer to the System and open the operating software ND-100.
- Selected the option Nucleic acid.
- With the sampling arm open, pipetted 1µl distilled water onto the lower measurement pedestal.
- Closed the sampling arm and initiated a spectral measurement using the operating software on the PC. The sample column is automatically drawn between the upper and lower measurement pedestals and the spectral measurement is made.
- Set the reading to zero with sample blank.
- 1µl sample was pipetted onto measurement pedestal and select measure.
- When the measurement was complete, opened the sampling arm and wiped the sample from both the upper and lower pedestals using a soft laboratory

wipe. Simple wiping prevents sample carryover in successive measurements for samples varying by more than 1000 fold in concentration.

3.3 Molecular markers used for the study

Three types of markers were used for the study which included ISSR (Inter Simple Sequences Repeats), SSR (Simple Sequence Repeats) and Sequenced characterised amplified region (SCAR).

3.3.1 DNA amplification conditions

The PCR condition required for effective amplification in ISSR, SSR and SCAR analysis included appropriate proportions of the component of the reaction mixture. The reaction mixture comprised of template DNA, assay buffer A or B, $MgCl_2$, Taq DNA polymerase, dNTPs and primers. The aliquot of this master mix were dispensed into 0.2ml PCR tubes. The PCR was carried out in Thermal Cycler (Applied Biosystem, USA or Agilent).

Another important factor, which affect amplification rate is the temperature profile or thermal cycle. The thermocycler was programmed for desired times and temperature for denaturation, annealing and polymerization based on the primer bases and method of assay.

3.3.2 ISSR (Inter Simple Sequence Repeat) analysis

The good quality genomic DNA (25-30 ng/µl) isolated from tomato leaf samples were subjected to ISSR analysis. ISSR primers with good resolving power were used for amplification of DNA.

PCR amplification was performed in a 20 µl reaction mixture and the composition of the reaction mixture consisted of,

a) Genomic DNA (30 ng) - 2.0 µl

b) 10X Taq assay buffer B - 2.0 µl

c) $MgCl_2$	- 2.0 µl
c) dNTP mix (10 mM each)	- 1.5 µl
d) Taq DNA polymerase (3U)	- 0.4 µl
e) Primer (10 pM)	- 1.5 µl
f) Autoclaved distilled water	- 10.6 µl
Total volume	- 20.0 µl

The amplification was carried out with the following programme

94^0C for 5 minutes	-	Initial denaturation	
94^0C for 1 minute	-	Denaturation	35 cycles
32^0C to 57.5^0C for 1 minutes	-	Primer annealing	
72^0C for 2 minutes	-	Primer extension	
72^0C for 10 minutes	-	Final extension	

4^0C for infinity to hold the sample

3.3.2.1 Screening of ISSR primers and analysis

Primers were selected from literature based on previous studies in ISSR analysis in tomato. Details of the primers bases provided in Table 2.

The amplified products were run on 1.5 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (100-bp DNA ladder). The profile was visualized under gel documentation unit and analysed for further analysis. The documented ISSR profiles were carefully examined for amplification of bands.

Table 2: List of ISSR primers screened with parents DNA samples

Sr. No.	Name of Primer	Nucleotide sequence	No. of bases
01	ISSR 2	CACACACACACACACAAA GC T	21
02	ISSR KAU 4	ATTATTATTATTATTGTT	18
03	ISSR KAU 7	ATT ATT GTT GTTGTT GTA	18
04	ISSR 7	AGAGAGAGAGAGAGAGCTG	19
05	ISSR KAU 9	TTA TTATTATTATTATTA CT	20
06	ISSR 1	GTT GTTGTTGTTGTT	15
07	ISSR 2	CTGA CTGACTGACTGA	16
08	ISSR 3	CCA CCACCACCACCA	15
09	ISSR 4	CTC CTCCTCCTCCTC	15
10	17899 A	CA CACACACACA AG	14
11	HB 10	GA GAGAGAGAGA CC	14
12	HB 12	CAC CAC CAC GC	11
13	UBC 823	T CT CTCTCTCTCTCT CC	17
14	UBC 835	AG AGAGAGAGAGAGAGAG	18

3.3.3 SSR (Simple Sequence Repeat) and SCAR (Sequence Characterised Amplified Region) analysis

The good quality genomic DNA (20 to 25ng/µl) isolated from tomato leaf samples (F_3 population). SSR and SCAR primers supplied by sigma, USA were used for amplification of DNA. So SSR and SCAR primers for the assay were selected after an initial screening of primers with parents (Sakthi and IIHR 2196).

The amplification was carried out in Thermal Cycler (Applied Biosystem, USA) or Agilent. PCR amplification was performed in a 20 µl reaction mixture which consisted of,

a) Genomic DNA (30ng)	-	2.0 µl
b) 10X Taq assay buffer A	-	2.0 µl
c) dNTP mix (10mm each)	-	1.5 µl
d) Taq DNA Polymerase (1U)	-	0.3 µl
e) Forward Primer (10pM)	-	0.75 µl
f) Reverse Primer (10pM)	-	0.75 µl
g) Autoclaved Distilled Water	-	12.7 µl
Total volume	-	20.0 µl

The thermocycler was carried out with the following programme

94^0C for 10 minutes	-	Initial denaturation	
94^0C for 1 minute	-	Denaturation	35 cycles
48.1^0C to 62.1^0C for 1 minute	-	Primer annealing	
72^0C for 2 minutes	-	Primer extension	
72^0C for 10 minutes	-	Final extension	
4^0C for infinity to hold the sample			

3.3.3.1 Screening of SSR and SCAR primers

The amplified products were run on two per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (100bp ladder). The profile was visualized under gel documentation unit and documented. The documented SSR profiles were carefully examined for amplification of DNA as bands. The details of the SSR SCAR primes bases are provided in Table 3 and 4 respectively.

Table 3: List of SSR primers screened with parents DNA samples

Sl No	Name of Primer	Nucleotide sequence		No. of bases
1	SSR 450	F	5’ AAT GAA GAA CCA TTC CGC AC 3’	20
		R	5’ ACA TGA GCC CAA TGA ACC TC 3’	20
2	SSR 306	F	5’ ACA TGA GCC CAA TGA ACC TC 3’	20
		R	5’ AAC CAT TCC GCA CGT ACA TA 3’	20
3	SSR 285	F	5’ AGT GGC TCT CAC CTA CTG CG 3’	20
		R	5’ CAA TTC TCA GGC ATG AAA CG 3’	20
4	SSRKAU 11	F	5’ TGT TGG TTG GAG AAA CTC CC 3’	20
		R	5’ AGG CAT TTA AAC CAA TAG GTA GC 3’	23
5	SSR 350	F	5’ GGA ATA ACC TCT AAC TGC GGG 3’	20
		R	5’ CGA TGC CTT CAT TTG GAC TT 3’	21
6	SSR 301	F	5’ TTT CCA CCT CAA ACC ACT CC 3’	20
		R	5’ CCC TTT GAC CTG TGC CA 3’	17
7	LEaat 002	F	5’ GCG AAG AAG ATG AGT CTA GAG CAT AG 3’	26
		R	5’ CTC TCT CCC ATG AGT TCT CCT CTT C 3’	25
8	LEat 006	F	5’ CAT AAT CAC AAG CTT CTT TCG CCA 3’	24
		R	5’ CAT ATC CGC TCG TTT CGT TAT GTA AT 3’	26
9	LEaat 007	F	5’ CAA CAG CAT AGT GGA GGA GG 3’	20
		R	5’ TAC ATT TCT CTC TCT CCC ATG AG 3’	23
10	LEaat 014	F	5’ TGT GTT GCG TCA TTA CCA CTA AAC 3’	24
		R	5’ CCC AAC CAC CAA TAC TTT CC 3’	20
11	LEaat 08	F	5’ GAG TCA ACA GCA TAG TGG AGG AGG 3’	24
		R	5’ CGT CGC AAT TCT CAG GCA TG 3’	20
12	LEaat 001	F	5’ GAT GGA CAC CCT TCA ATT TAT GGT 3’	24
		R	5’ TCC AAG TAT CAG GCA CAC CAG C 3’	22
13	LEaat 15	F	5’ GGA TTG TAG AGG TGT TGT TGG 3’	21
		R	5’ TTT GTA ATT GAC TTT GTC GAT G 3’	21

contd…

Sl No	Name of Primers	Nucleotide sequence		No. of bases
14	LEaat 16	F	5’ CCC AAA TGC TAT GCA ATA CAC 3’	21
		R	5’ AGT TCA GGA TTG GTT TAA GGG 3’	21

Table 4: List of SCAR primers screened with parents DNA samples

Sl No	Name of Primers	Nucleotide sequence		Number of bases
1	TSCAR AAT/CGA	F	5‘ TAG ATG GAA TCC AAT ATC AGG 3‘	21
		R	5’ AAC CAC AGT GAA GGA ATA TAC A 3’	22
2	TSCAR AAG/CAT	F	5’ AGA AGG TCA CGG CGA GA 3’	17
		R	5’ TGA GTC CTG AGT AAC TGG 3’	18
3	Ualty3a	F	5’GAC CTT CAA AAT GAT CAG ATA 3’	21
		R	5’ TGG ACC CTT TTT ACC CTA AGC 3’	21
4	Ualty3b	F	5’ CTC CAC AGC TTC AAT GCA AA 3’	20
		R	5’ CGT GAA TAC CTT GAT TCT TGA 3’	21
5	Ualty 5	F	5’ TAG GAA ATG TTG AAC TAT TGT GTT 3‘	24
		R	5’ TCA TGC GAT GAA GAG GTC TAT G 3‘	22
6	Ualty 6	F	5’ TGT TGT GAT TGT TAT TGT CAA C 3‘	22
		R	5’ CTG GCA AGC GTG TAA CTC AC 3’	20
7	Ualty 11	F	5’ TTA ATT CTA GGG ATT TGG CAG T 3’	22
		R	5’ CCC AAG CCA TCA TGA GAT TC 3’	20
8	Ualty 16	F	5’ GCA CAA AAA TGC TTT TGG ACA 3’	21
		R	5’ TTC CGA ATT AAC AGA GTC TCC AC 3’	23

Results

4. RESULTS

The observations recorded and the results obtained in the study "Integration of combined disease resistance for bacterial wilt and ToLCV in tomato (*Solanum lycopersicum* L.) through marker assisted selection" are presented below:

4.1 Evaluation of parents and F_3 population for bacterial wilt and ToLCV

4.1.1 Evaluation of parents Sakthi and IIHR 2196

Sakthi (Bacterial wilt resistant) as female parent and IIHR 2196 (ToLCV resistant) as male parent were selected for developed the combined disease resistance in tomato. Five plants from each parent were selected for biometric character and disease reaction. The details of biometric character of parents are given in Table 5. Sakthi was observed to be resistant to bacterial wilt and IIHR 2196 was resistant to ToLCV.

4.1.2 Screening of F_3 population from selected F_2 lines

Four mother plants selected from F_2 population used for the study, included F_2-06, F_2-18, F_2-20 and F_2-33 having both bacterial wilt and ToLCV resistance. Sixty seeds from each mother plant were used for raising F_3 population. Two hundreds F_3 plants were screened for bacterial wilt and ToLCV resistance during October 2013-January 2014 under field condition. The biometric characters of F_3 population are given in table 6. Four types of fruit shaped were observed in F_3 population (Plate 5) and few plants were showed both bacterial wilt and ToLCV resistance (Plate 6). Four mother plants were classified based on disease reaction of progenies to bacterial wilt and ToLCV (Table 7 and 8). All F_2 lines showed moderately susceptible with respect to bacterial wilt resistance. These lines classified based on per cent disease incidence as suggested by Mew and Ho (1976) for bacterial wilt disease. F_2-06 and F_2-20 lines observed highly susceptible and F_2-18 and F_2-33 were observed susceptible for ToLCV resistance.

a. Slightly flattened

b. Circular

c. Cylindrical

d. Pear shaped

Plate 5: Different fruit shapes observed in F_3 population

Plate 6: Tomato F_3 line (F_2-33-37) with combined resistance to bacterial wilt and ToLCV

Table- 5 Biometric characters of parents Sakthi (Bacterial wilt resistant) and IIHR 2196 ToLCV resistant (January 2013-April 2013).

Sl. No.	Plant No.	Plant height (cm)	Days to flowering	Days to fruiting	Growth habit	Fruit size and shape	Fruits /plant	Yield/ plant (g)	Average fruit weight (g)	Disease reaction	
										BW	ToLCV
Sakthi											
01	Sakthi -01	59	23	34	SD	C	07	166	23.7	R	
02	Sakthi -02	62	24	36	SD	C	08	179	22.3	R	
03	Sakthi -03	32	22	35	SD	C	05	116	23.2	R	
04	Sakthi -04	47	23	36	SD	C	09	140	15.5	R	
05	Sakthi -05	56	26	39	SD	C	11	146	13.2	R	
IIHR 2196											
06	IHR 2196-01	69	27	41	I	Cy	12	258	21.5		R
07	IHR 2196-02	52	23	36	I	Cy	09	298	33.1		R
08	IHR 2196-03	64	26	41	I	Cy	24	776	32.3		R
09	IHR 2196-04	72	21	32	I	Cy	13	524	40.3		R
10	IHR 2196-05	67	26	43	I	Cy	22	288	13		R

Table- 6 Biometric characters and disease reaction of F_3 population (F_2-06, F_2-18, F_2-20 and F_2-33) observed in field condition (October 2013 - Jaunary 2014).

Sl. No.	Plant No.	Plant height (cm)	Days to flowering	Days to fruiting	Growth habit	Fruit size and shape	Fruits /plant	Yield/ plant (g)	Average fruit weight (g)	Disease reaction in field	
										BW	ToLCV
F_2-06											
01	F_2-06-01	30	27		D		00	0	0	R	S
02	F_2-06-02	57	30	47	D	SF	12	286	23.8	R	R
03	F_2-06-04	64	25	40	SD	SF	33	1180	35.7	R	S
04	F_2-06-05	72	25	44	SD	SF	20	348	17.4	R	S
05	F_2-06-06	64	24	36	SD	C	13	484	37.2	R	S
06	F_2-06-08	76	20	37	SD	SF	05	180	36	R	S
07	F_2-06-09	74	21	33	SD	Cy	29	498	17.1	R	S
08	F_2-06-10	59	23	34	D	SF	07	166	23.7	R	S
09	F_2-06-11	67	23	33	SD	SF	14	294	21	R	S
10	F_2-06-12	63	23	35	SD	SF	10	372	37.2	R	S
11	F2-06-15	39	23	37	D	SF	04	92	23	R	S
12	F2-06-16	73	22	36	SD	C	18	604	33.5	R	S
13	F2-06-20	59	23	35	D	Cy	06	130	21.6	R	S
14	F2-06-22	69	22	35	SD	C	08	96	12	R	R
15	F2-06-24	68	22	33	SD	SF	21	520	24.7	R	S
16	F2-06-25	46	23	35	D	Cy	10	130	13.6	R	S
17	F2-06-26	40	31	46	D		00	0	0	R	S
18	F2-06-27	79	23	36	SD	Cy	29	730	25.1	R	S
19	F2-06-28	51	22	33	D	SF	16	252	15.7	R	S

Biometric characters and disease reaction of F_3 population (F_2-06, F_2-18, F_2-20 and F_2-33) observed in field condition contd…

Sl. No.	Plant No.	Plant height (cm)	Days to flowering	Days to fruiting	Growth habit	Fruit size and shape	Fruits /plant	Yield/ plant (g)	Average fruit weight (g)	Disease reaction in field	
										BW	ToLCV
20	F2-06-29	56	23	38	D	SF	03	72	24	R	S
21	F2-06-30	73	21	36	SD	SF	15	350	23.3	R	S
22	F2-06-38	36	23	36	D	C	14	122	8.7	R	S
23	F2-06-39	39	22	0	D	C	00	0	0	R	S
24	F2-06-45	43	23	39	D		00	0	0	R	S
25	F2-06-47	34	23	35	D	Cy	16	314	19.6	R	S
26	F2-06-50	57	25	38	D	Cy	03	62	20.6	R	S
F_2-18											
01	F2-18-01	61	22	34	SD	Cy	17	344	20.2	R	S
02	F2-18-06	60	22	34	D	Cy	05	104	20.8	R	S
03	F2-18-08	55	22	38	D	SF	01	36	36	R	R
04	F2-18-10	77	23	37	SD	Cy	11	390	35.4	R	S
05	F2-18-11	49	27	41	D		00	0	0	R	S
06	F2-18-14	55	23	37	D	Cy	03	58	19	R	R
07	F2-18-18	76	23	39	SD	C	17	128	7.5	R	S
08	F2-18-19	34	22	37	D		00	0	0	R	S
09	F2-18-21	46	28	47	D		00	0	0	R	S
10	F2-18-22	76	22	36	SD	Cy	15	350	23.3	R	R
11	F2-18-24	92	22	36	I	Cy	07	210	30	R	S
12	F2-18-25	75	34	33	SD	Cy	07	164	23.4	R	S
13	F2-18-27	57	23	36	D	C	02	41	20.5	R	S
14	F2-18-28	64	29	47	D	C	05	94	18.8	R	S

Biometric characters and disease reaction of F_3 population (F_2-06, F_2-18, F_2-20 and F_2-33) observed in field condition contd…

Sl. No.	Plant No.	Plant height (cm)	Days to flowering	Days to fruiting	Growth habit	Fruit size and shape	Fruits /plant	Yield/ plant (g)	Average fruit weight (g)	Disease reaction in field	
										BW	ToLCV
15	F2-18-30	95	22	34	I	Cy	19	448	23.5	R	S
16	F2-18-31	79	21	33	SD	SF	12	620	51.6	R	S
17	F2-18-32	83	23	41	SD	SF	30	872	29	R	S
18	F2-18-34	69	24	35	SD	Cy	07	222	31.7	R	S
19	F2-18-35	62	29	34	SD	SF	08	322	40.2	R	S
20	F2-18-36	32	21		D		00	0	0	R	S
21	F2-18-37	35	23	32	D	Cy	09	226	25.1	R	S
22	F2-18-38	87	22	36	SD	Cy	14	457	32.6	R	S
23	F2-18-39	35	32		D		00	0	0	R	R
24	F2-18-40	33	35	47	D		00	0	0	R	S
25	F2-18-42	70	21	41	SD	R	22	878	39.9	R	S
26	F2-18-45	44	23		D		00	0	0	R	S
27	F2-18-49	88	22	34	SD	SF	22	1096	49.8	R	S
28	F2-18-50	39	43		D		00	0	0	R	S
F_2-20											
01	F2-20-01	54	25	45	D	SF	07	200	28.5	R	S
02	F2-20-02	74	23	37	SD	PS	10	146	14.6	R	S
03	F2-20-04	56	22	39	D	PS	03	60	20	R	S
04	F2-20-05	49	23	41	D		00	0	0	R	R
05	F2-20-06	50	23	36	D	PS	01	20	20	R	S
06	F2-20-07	59	43		D		00	0	0	R	S

Biometric characters and disease reaction of F_3 population (F_2-06, F_2-18, F_2-20 and F_2-33) observed in field condition contd…

Sl. No.	Plant No.	Plant height (cm)	Days to flowering	Days to fruiting	Growth habit	Fruit size and shape	Fruits /plant	Yield/ plant (g)	Average fruit weight (g)	Disease reaction in field	
										BW	ToLCV
07	F2-20-13	64	23	37	SD	PS	02	20	10	R	S
08	F2-20-19	70	22	36	SD	Cy	11	190	17.4	R	S
09	F2-20-21	62	23	37	D	PS	09	260	28.8	R	S
10	F2-20-22	36	24	38	D	PS	03	24	8	R	S
11	F2-20-23	45	29	46	D		00	0	0	R	S
12	F2-20-24	65	27	46	D	PS	07	114	16.2	R	S
13	F2-20-26	32	27	40	D		00	0	0	R	S
14	F2-20-29	80	22	36	SD	PS	17	164	9.6	R	S
15	F2-20-30	40			D		00	0	0	R	S
16	F2-20-32	37	23	36	D		00	0	0	R	S
17	F2-20-33	37	25	47	D		00	0	0	R	S
18	F2-20-34	35	25	37	D		00	0	0	R	S
19	F2-20-40	30	25		D		00	0	0	R	R
F_2-33											
01	F2-33-02	35	23	33	D	C	03	66	22	R	S
02	F2-33-03	59	21	36	D	C	27	622	23	R	S
03	F2-33-08	69	25	42	D	SF	17	366	21.5	R	S
04	F2-33-09	56	28	40	D	SF	02	69	34.5	R	S
05	F2-33-12	54	16	23	D	SF	13	364	28	R	S
06	F2-33-17	89	23	41	SD	SF	42	1170	27.8	R	S
07	F2-33-21	38	21		D		00	0	0	R	S
08	F2-33-22	65	31	36	D	SF	49	2540	51.8	R	S

Biometric characters and disease reaction of F_3 population (F_2-06, F_2-18, F_2-20 and F_2-33) observed in field condition contd…

Sl. No.	Plant No.	Plant height (cm)	Days of flowering	Days of fruiting	Growth habit	Fruit size and shape	Fruits /plant	Yield/ plant (g)	Average fruit weight (g)	Disease reaction	
										BW	ToLCV
09	F2-33-23	72	22	35	SD	C	65	2575	39.6	R	R
10	F2-33-24	49	23	35	D	SF	26	723	27.8	R	S
11	F2-33-27	57	23	31	D	SF	38	1410	37.1	R	S
12	F2-33-30	56	23	39	D	SF	92	2874	31.2	R	R
13	F2-33-31	72	25	40	SD	SF	49	1330	27.1	R	S
14	F2-33-32	64	23	34	D	SF	72	2358	32.7	R	S
15	F2-33-35	80	22	35	I	SF	52	1489	28.6	R	S
16	F2-33-37	98	22	31	I	SF	84	3281	34.9	R	R
17	F2-33-38	62	22	32	D	C	91	2614	28.7	R	R
18	F2-33-40	45	27	46	D	SF	16	292	18.2	R	S
19	F2-33-42	70	23	36	SD	C	29	558	19.2	R	S
20	F2-33-45	75	23	39	SD	C	30	830	27.6	R	S
21	F2-33-46	95	23	39	I	SF	86	3006	34.9	R	S
22	F2-33-50	64	23	39	D	SF	22	926	42	R	S

D- Determinate, SI- Semi determinate, I- Indeterminate, C- Circular, Cy- Cylindrical, SF- Slightly flattened, PS- Pear shaped, Re- Rectangular, BW- Bacterial wilt, ToLCV- Tomato leaf curl virus, R- Resistance, S-Susceptible.

Table- 7 Reaction of F_3 segregants to bacterial wilt in field

Sl. No.	Mother plants	No. of plants evaluated	No. of plants infected	PDI (%)	Disease Reaction
1	F_2-06	50	20	40	Moderately Susceptible
2	F_2-18	50	22	44	Moderately Susceptible
3	F_2-20	50	28	56	Moderately Susceptible
4	F_2-33	50	26	52	Moderately Susceptible

Table- 8 Reaction of F_3 segregants to ToLCV in field

Sl. No.	Mother plants	No. of plants evaluated	No. of plants infected	PDI (%)	PDS (%)	C.I.	Category
1	F_2-06	30	28	93.33	74.16	69.21	Highly Susceptible
2	F_2-18	28	24	85.71	69.64	59.68	Susceptible
3	F_2-20	22	20	90.90	86.36	78.50	Highly Susceptible
4	F_2-33	24	20	83.33	65.62	54.68	Susceptible

These lines were classified based on coefficient of infection as suggested by Banerjee and Kalloo (2000) for ToLCV disease.

4.2 Evaluation of F_3 population for yield and its components

4.2.1 Plant height

The plant height ranged from 32 to 62 in the parent Sakthi and 52 to 72 in IIHR 2196 (Table 9). The F_3 population of the selected F_2 parents varied with respect to plant height. Analysis of variance indicated superiority of F_2-18 with respect to plant height. Maximum plant height was recorded in the progenies of F_2-33 (98 cm). The minimum plant height was observed in progenies of F_2-06 and F_2-20 (30 cm). The highest mean value for plant height was recorded in the F_2-18 (60.93 cm) and lowest mean value was observed in the F_2-20 (49.0 cm).

4.2.2 Days to flowering

Days to flowering ranged from 22 to 26 days in Sakthi and 21 to 27 days in IIHR 2196 (Table 10). No significant variation was observed among F_3 population. The lowest mean value for days to flowering was recorded in F_2-06 (23.56 days) and the highest mean value was recorded in F_2-18 (25.21 days).

4.2.3 Days to fruiting

Days to fruiting in the parents ranged from 34 to 39 days for Sakthi and 32 to 43 days in IIHR 2196 (Table 11). There was no significant variation among the F_3 population. In the F_3 population the lowest mean value for days to fruiting was observed in F_2-18 (36.07 days) and highest mean value was recorded in F_2-20 (39.35 days).

4.2.4 Number of fruits per plant

The number of fruits per plant ranged from 05 to 11 in Sakthi and 09 to 24 in IIHR 2196 (Table 12). The F_3 progenies of the selected F_2 parents varied with

Table- 9 Mean performance of F_3 progenies for plant height

Sl. No.	Parents and Mother plants	No. progenies evaluated	Range	Minimum	Maximum	Mean
1	Sakthi	05	30	32	62	51.23
2	IIHR 2196	05	20	52	72	64.85
3	F_2-06	30	49.00	30.00	79.00	55.33^{ab}
4	F_2-18	28	63.00	32.00	95.00	60.93^{a}
5	F_2-20	22	50.00	30.00	80.00	49.00^{b}
6	F_2-33	24	63.00	35.00	98.00	58.57^{ab}

a;b- Duncan score of the DMRT analysis

Table- 10 Mean performance of F_3 progenies for days to flowering

Sl. No.	Parents and Mother plants	No. progenies evaluated	Range	Minimum	Maximum	Mean
1	Sakthi	05	04	22	26	23.63
2	IIHR 2196	05	06	21	27	24.64
3	F_2-06	30	11.00	20.00	31.00	23.56^{a}
4	F_2-18	28	22.00	21.00	43.00	25.21^{a}
5	F_2-20	22	21.00	22.00	43.00	25.05^{a}
6	F_2-33	24	15.00	16.00	31.00	23.70^{a}

a- Duncan score of the DMRT analysis

Table- 11 Mean performance of F_3 progenies for days to fruiting

Sl. No.	Parents and Mother plants	No. progenies evaluated	Range	Minimum	Maximum	Mean
1	Sakthi	05	05	34	39	36.00
2	IIHR 2196	05	11	32	43	38.61
3	F_2-06	30	14	33	47	37.03^{a}
4	F_2-18	28	15	32	47	36.07^{a}
5	F_2-20	22	11	36	47	39.35^{a}
6	F_2-33	24	23	23	46	37.48^{a}

a- Duncan score of the DMRT analysis

respect to number of fruits per plant. Analysis of variance indicated superiority for F_2-33 with respect to number of fruits per plant (41.36). F_2-33-30 plant gave the 92 fruits among the 104 progenies. This group also recorded the highest mean value for number of fruits per plant (41.36). The lowest mean value was observed in progenies of F_2-20 (07.00). In F_3 population of some plants not able to produced fruits due to high per cent disease infection include ToLCV, early blight and curly top virus.

4.2.5 Yield per plant

The yield per plant varied from 116 to 179 g in Sakthi and 258 to 776 g in IIHR 2196 (Table 13). The F_3 population also showed variation with respect to yield per plant. Analysis of variance indicated superiority for F_2-33 (1347.27 g) with respect to yield per plant. The lowest mean value was recorded by F_2-20 (119.08 g).

4.2.6 Average fruit weight

The average fruit weight ranged from 13.2 to 23.7 g in Sakthi and 13.0 to 40.0g in IIHR 2196 (Table 14). The F_3 progenies of the selected F_2 parents varied with respect to average fruit weight. Analysis of variance indicated superiority for F_2-33 with respect to average fruit weight. The highest average fruit weight was recorded by F_3 population of F_2-33 (30.36 g) and lowest mean value was observed in F_2-20 (17.31 g).

4.3 Screening of parents (Sakthi and IIHR 2196) with reported primers for BW and ToLCV resistance

4.3.1 Isolation, purification and quantification of DNA

Two tomato genotypes selected as parents includes, Sakthi which is reported to have bacterial wilt resistance and IIHR 2196 which is having ToLCV resistance. The genomic DNA isolated through the CTAB method reported by

Table- 12 Mean performance of F_3 progenies for fruits per plant

Sl. No.	Parents and Mother plants	No. progenies evaluated	Range	Minimum	Maximum	Mean
1	Sakthi	05	06	05	11	02.23
2	IIHR 2196	05	15	09	24	16.00
3	F_2-06	30	30	03	33	13.90[b]
4	F_2-18	28	29	01	30	11.65[b]
5	F_2-20	22	16	01	17	07.00[b]
6	F_2-33	24	90	02	92	41.36[a]

a;b- Duncan score of the DMRT analysis

Table- 13 Mean performance of F_3 progenies for yield per plant

Sl. No.	Parents and Mother plants	No. progenies evaluated	Range	Minimum	Maximum	Mean
1	Sakthi	05	63	116	179	149.41
2	IIHR 2196	05	518	258	776	428.82
3	F_2-06	30	1118	62	1180	331.09[b]
4	F_2-18	28	1060	36	1096	357.00[b]
5	F_2-20	22	240	20	260	119.08[b]
6	F_2-33	24	3215	66	3281	1347.27[a]

a;b- Duncan score of the DMRT analysis

Table- 14 Mean performance of F_3 progenies for average fruit weight

Sl. No.	Parents and Mother plants	No. progenies evaluated	Range	Minimum	Maximum	Mean
1	Sakthi	05	10.5	13.2	23.7	19.58
2	IIHR 2196	05	27.3	13.0	40.3	28.04
3	F_2-06	30	28.5	08.7	37.2	23.39[a]
4	F_2-18	28	44.1	07.5	51.6	28.91[a]
5	F_2-20	22	20.8	08.0	28.8	17.31[b]
6	F_2-33	24	33.6	18.2	51.8	30.36[a]

a;b- Duncan score of the DMRT analysis

Roger and Bendich (1994) were pure with less RNA contamination (Plate.7). The agarose gel electrophoresis indicated clear discrete band without contamination of RNA and proteins. Spectrophotometric analysis gave the ratio of UV absorbance ($A_{260/280}$) between 1.8 and 2.0.

4.3.2 Screening parents Sakthi and IIHR 2196 reported primers

4.3.2.1 ISSR primers screening

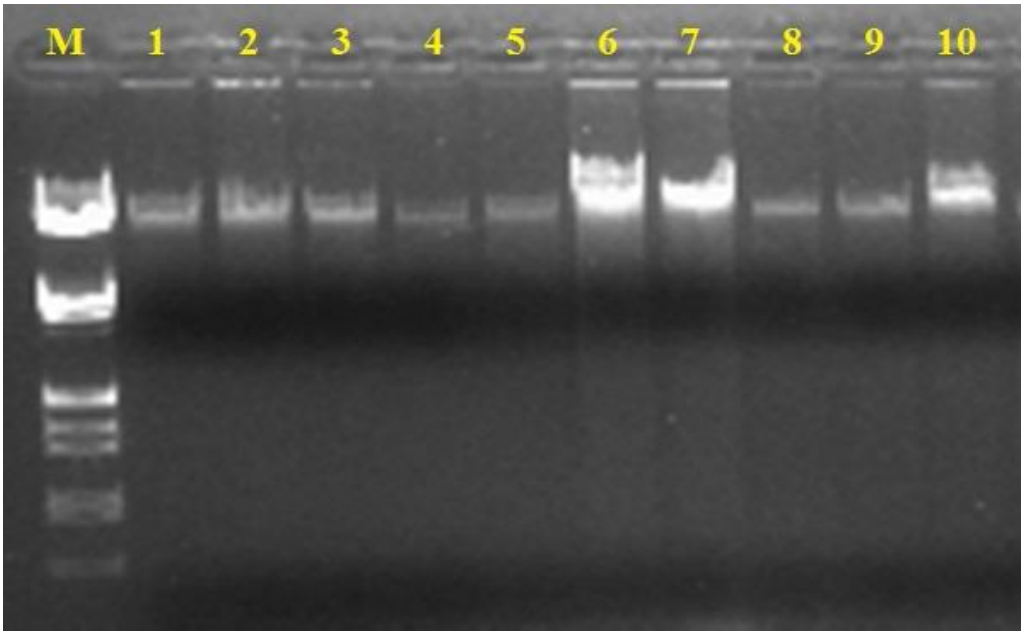
Five ISSR primers reported earlier were screened with parental DNA which are specific to bacterial wilt and ToLCV. Out of five primers no one primer showed polymorphic band (Plate. 8a) in parents. The primers reported earlier failed to give polymorphic amplicons even when tried with stringent PCR condition reported and also when varying annealing temperature were tried. Then another nine primers were screened with parental DNA which is not specific to bacterial wilt and ToLCV. Out of nine primers one primer was shown the polymorphic band in IIHR 2196 which is resistant to ToLCV (Plate. 8b). ISSR HB 12 primer was selected for screening the F_3 population. The sequence data of the primers selected for screening is already provided in Table 2 under section 3.3.2.1.

HB 12:

Primer HB 12 amplified with Sakthi gave six amplicons and in IIHR 2196 gave seven amplicons. From those seven bands one was polymorphic in IIHR 2196. The pattern of amplification is shown in Plate 8b. The molecular weight of the polymorphic band was 1500 bp.

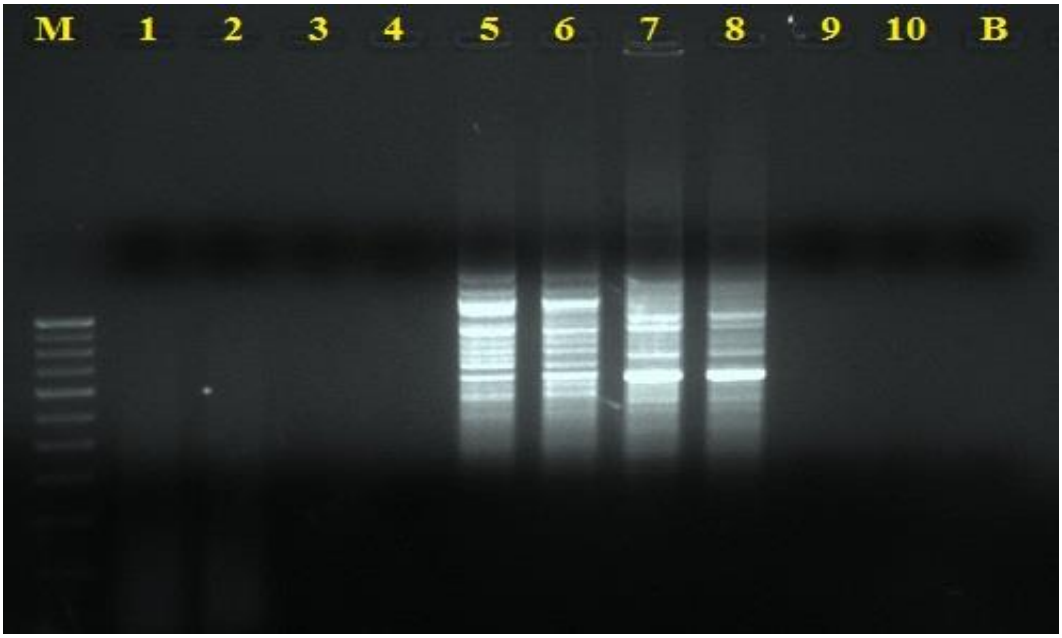
4.3.2.2 SSR primers screening

Fourteen SSR primers were selected for screening of parents which are reported as specific to bacterial wilt and ToLCV. Out of these, three primers (SSRKAU 11, SSR 450 and LEaat 16) gave polymorphic banding pattern in Sakthi and IIHR 2196. The pattern of amplification is shown in Plate 9. The



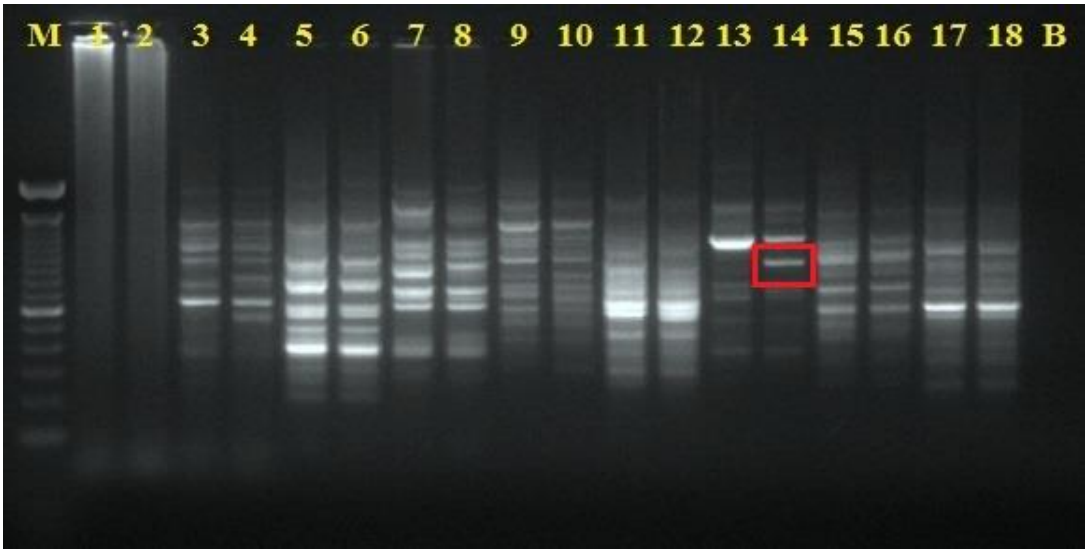
M- Marker (1 Kb), well number/Lane 1 to 5- Sakthi and 6 to 10- IIHR 2196

Plate 7: Intact DNA isolation through CTAB method from parents (Sakthi and IIHR 2196)



M- Marker (1 Kb), 1- ISSRKAU 2 with Sakthi, 2- ISSRKAU 2 with IIHR 2196, 3- ISSRKAU 4 with Sakthi, 4- ISSRKAU 4 with IIHR 2196, 5- ISSRKAU 07 with Sakthi, 6- ISSRKAU 07 with IIHR 2196, 7- ISSR 7 with Sakthi, 8- ISSR 7 with IIHR 2196, 9- ISSRKAU 9 with Sakthi, 10- ISSRKAU 9 with IIHR 2196

Plate 8a: Screening of ISSR primers (ISSR 2, 4, 07, 7 AND 9) with the parents Sakthi and IIHR 2196



M- Marker (1-3 Kb), 1- ISSR 1 with Sakthi, 2- ISSR 1 with IIHR 2196, 3- ISSR 2 with Sakthi, 4- ISSR 2 with IIHR 2196, 5- ISSR 3 with Sakthi, 6- ISSR 3 with IIHR 2196, 7- ISSR 4 with Sakthi, 8- ISSR 4 with IIHR 2196, 9- 17899 A with Sakthi, 10- 17899 A with IIHR 2196, 11- HB 10- with Sakthi, 12- HB 10 with IIHR 2196, 13- HB 12 with Sakthi, 14- HB 12 with IIHR 2196, 15- UBC 823 with Sakthi, 16- UBC 823 with IIHR 2196, 17- UBC 835 with Sakthi, 18- UBC 835 with IIHR 2196, B- Blank (Without sample)

Plate 8b: Screening of ISSR primers with the parents Sakthi and IIHR 2196

sequence data of primes selected for screening is given in Table 3 under the section of 3.3.3.1. The amplification patterns of selected primer are as follows.

SSRKAU 11:

Primer SSRKAU 11 gave two bands in both Sakthi and IIHR 2196. Those two bands have shown slight polymorphism in Sakthi with respect to bacterial wilt and IIHR 2196 to ToLCV. The pattern of amplification is shown in Plate 9a. The size of polymorphic band was 270-280bp.

SSR 450:

This primer generated one band in both Sakthi and IIHR 2196 with slight polymorphism. The pattern of amplification is shown in Plate 9b. The molecular weight of the amplicon was 280-300 bp.

LEaat 16:

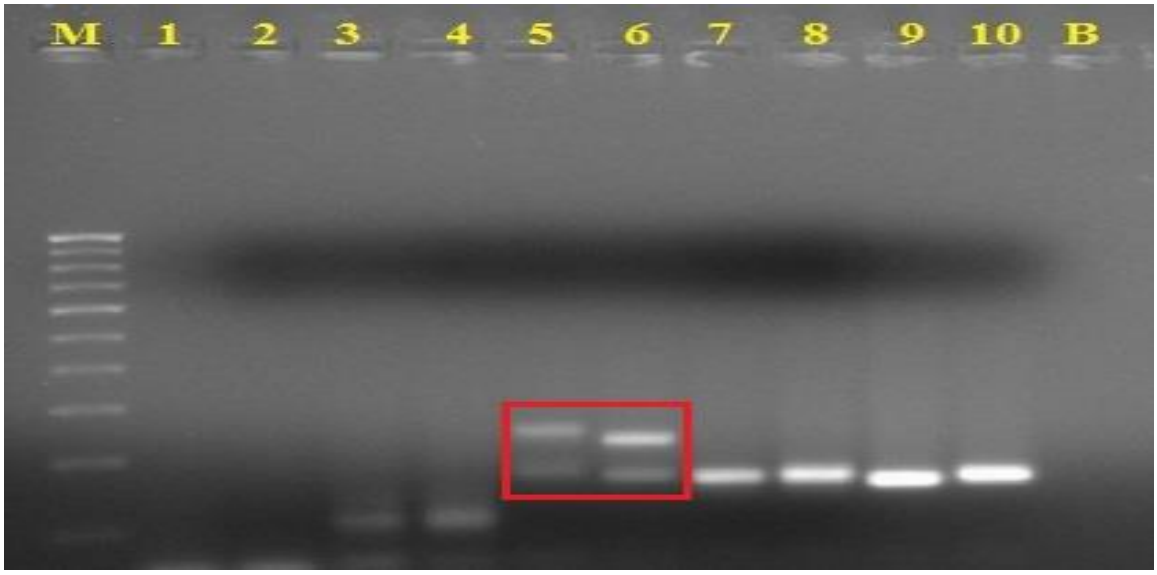
LEaat 16 primer gave specific amplicons of size 270 bp for bacterial wilt resistance in Sakthi and there was no amplification in IIHR 2196. The pattern of amplification is shown in Plate 9b.

4.3.2.3 SCAR primers screening

The parents (Sakthi and IIHR 2196) were used for SCAR primers screening. Eight SCAR primers were reported for specific to bacterial wilt and ToLCV resistance in tomato. Out of the eight primers only two primers (TSCAR $_{AAG/CAT}$ and Ualty 16) gave the polymorphic banding pattern in parents. The pattern of amplifications is shown in Plate 10a. The sequence data of primers selected for screening given in Table 4 under the section of 3.3.4.1.

TSCAR$_{AAG/CAT}$:

This primer is reported to be specific for bacterial wilt. When amplified, it generated two bands in Sakthi and one band in IIHR 2196. In Sakthi among the



M- Marker (1 Kb), 1- LEaat007 with Sakthi, 2- LEaat007 with IIHR 2196, 3- LEaat002 with Sakthi, 4- LEaat002 with IIHR 2196, 5- SSRKAU 11 with Sakthi, 6- SSRKAU 11 with IIHR 2196, 7- LEaat006 with Sakthi, 8- LEaat007 with IIHR 2196, 9- SSR 350 with Sakthi, 10- SSR 350 with IIHR 2196, B- Blank (Without Sample)

Plate 9a: Screening of SSR primers (LEaat 07, 02, 06, SSRKAU 11 and SSR 350) with the parents Sakthi and IIHR 2196

M- Marker (1-3 Kb), 1- SSR 285 with Sakthi, 2- SSR 285 with IIHR 2196, 3- SSR 301 with Sakthi, 4- SSR 301 with IIHR 2196, 5- SSR 306 with Sakthi, 6- SSR 306 with IIHR 2196, 7- SSR 450 with Sakthi, 8- SSR 450 with IIHR 2196, 9- LEaat 01 with Sakthi, 10- LEaat 01 with IIHR 2196, 11- LEaat 08 with Sakthi, 12- LEaat 08 with IIHR 2196, 13- LEaat 14 with Sakthi, 14- LEaat 14 with IIHR 2196, 15- LEaat 15 with Sakthi, 16- LEaat 15 with IIHR 2196, 17- LEaat 16 with Sakthi, 18- LEaat 16 with IIHR 2196, B- Blank (Without sample)

Plate 9b: Screening of SSR primers with the parents Sakthi and IIHR 2196

two bands, one was polymorphic and specific to bacterial wilt. The pattern of amplification is shown in Plate 10a. The molecular weight of the polymorphic amplicons was around 500 bp.

Ualty 16:

This primer is reported to be specific for ToLCV resistance. This primer when amplified with parents (Sakthi and IIHR 2196) generated one band in IIHR 2196 which is resistant to ToLCV. The pattern of amplification is shown in Plate 10a. The molecular weight of the amplicons was around 500 bp.

The details of amplicons and polymorphism observed with selected primers with respect to parents Sakthi and IIHR 2196 are provided in Table 15.

4.4 Screening of F_3 population with selected molecular markers

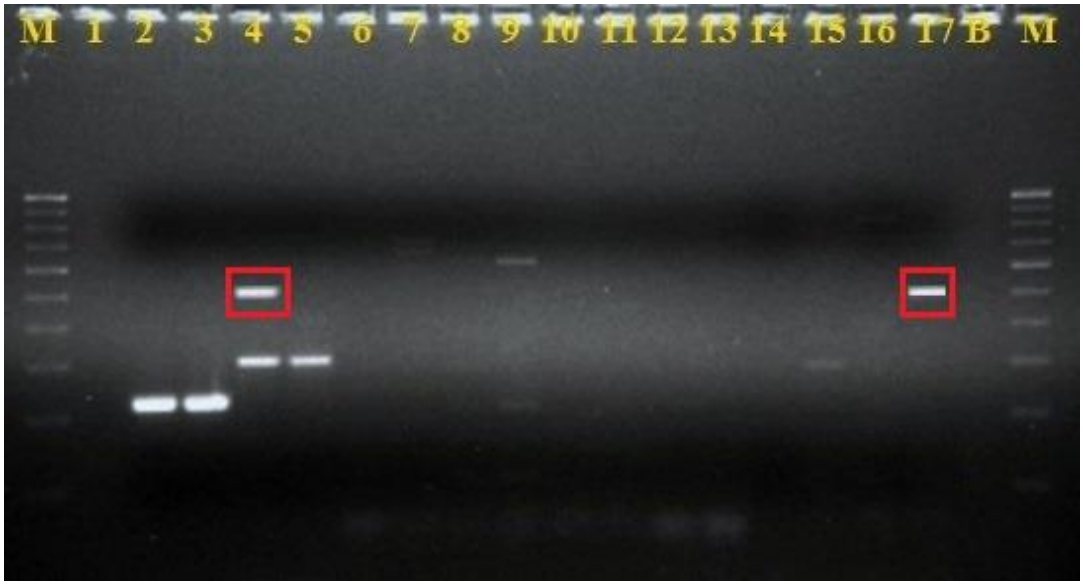
4.4.1 Isolation, purification and quantification of DNA

F_3 population (200 plants) was generated from selected four F_2 lines (F_2-06, F_2-18, F_2-20 and F_2-33) which were having bacterial wilt and ToLCV resistance. Genomic DNA from tender tomato leaves was isolated using CTAB method (Roger and Bendich, 1994) from all the 200 progenies. The DNA isolated was pure with slight RNA contamination. The agarose gel electrophoresis indicated clear discrete band without contamination of RNA and protein (Plate 10b). Spectrometric analysis gave ratio of UV absorbance ($A_{260/280}$) between 1.8 and 2.0.

4.4.2 ISSR analysis on F_3 population

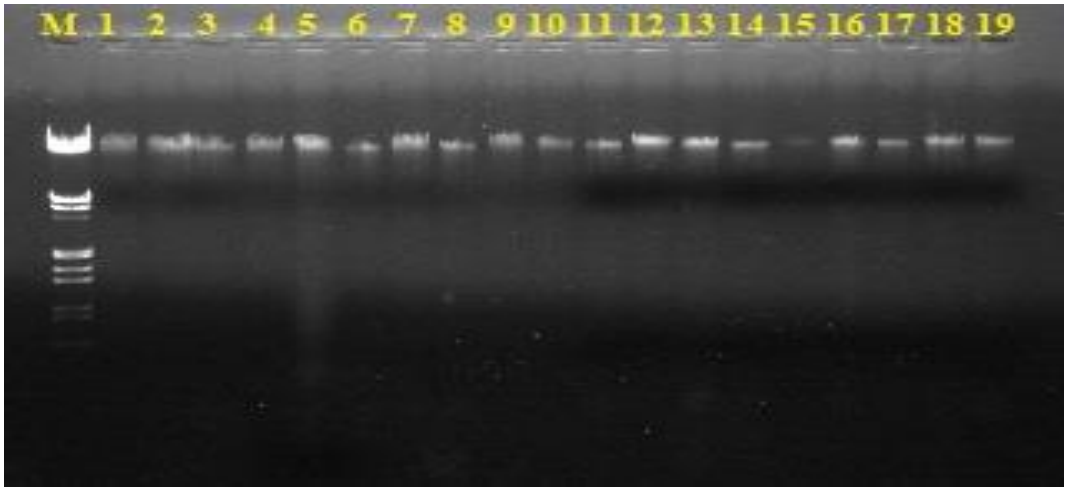
ISSR analysis was carried out for the two hundred plants with selected primer HB 12.

The amplification pattern of the primer HB 12 is provided in Plate 11. In F_3 population 134 progenies gave monomorphic and 66 gave polymorphic amplicons. The polymorphic amplicon size was 1500 bp. This was traced in the F_3



M- Marker (1 Kb), 1- Empty, 2- $SCAR_{AAT/CGA}$ with Sakthi, 3- $SCAR_{AAT/CGA}$ with IIHR 2196, 4- $SCAR_{AAT/CAT}$ with Sakthi, 5- $SCAR_{AAT/CAT}$ with IIHR 2196, 6- Ualty 3a with Sakthi, 7- Ualty 3a with IIHR 2196, 8- Ualty 3b with Sakthi, 9- Ualty 3b with IIHR 2196, 10- Ualty 5 with Sakthi, 11- Ualty 5 with IIHR 2196, 12- Ualty 6 with Sakthi, 13- Ualty 6 with IIHR 2196, 14- Ualty 11 with Sakthi, 15- Ualty11 with IIHR 2196, 16- Ualty 16 with Sakthi, 17- Ualty 16 with IIHR 2196, B- Blank (Without sample), M- Marker (1 Kb)

Plate 10a: Screening of SCAR primers with the parents Sakthi and IIHR 2196



M- Marker, Lane 1 to 18- F_3 plants

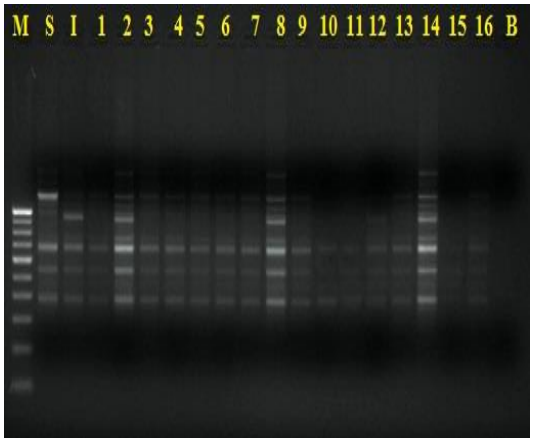
Plate 10b: Intact DNA isolated through CTAB method from F_3 segregants

Table- 15 Details of amplicons and polymorphism observed in selected primers with respect to parents Sakthi and IIHR 2196.

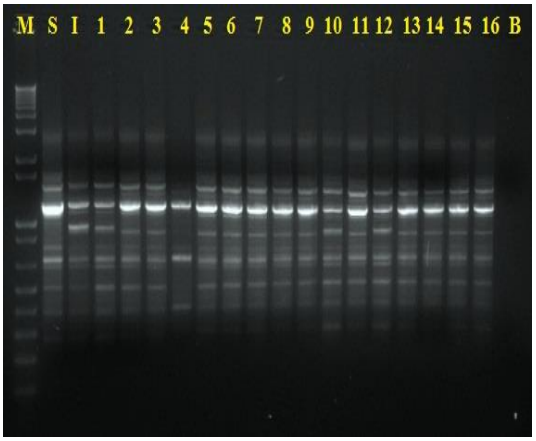
Primers details	Number of amplicons	Polymorphic amplicons	Size of polymorphic amplicons
ISSR			
HB 12	07	01	1500 bp
SSR			
SSRKAU 11	02	02	270 and 280 bp
SSR 450	02	02	280 and 300 bp
LEaat 16	01	01	270 bp
SCAR			
$TSCAR_{AAG/CAT}$	02	01	500 bp
Ualty 16	01	01	500 bp

Table- 16 Amplification pattern for the primer HB 12 in F_3 population

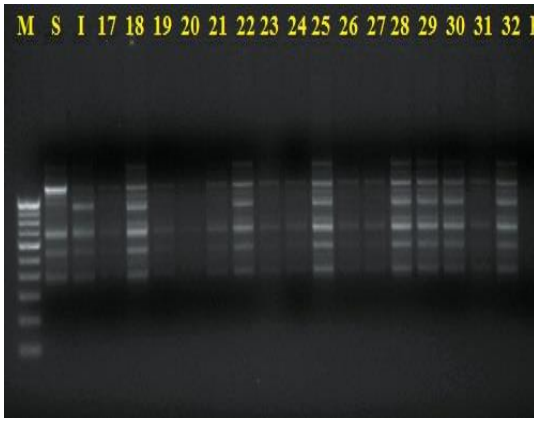
Population (Mother plants)	No. of F_3 progenies evaluated	No. of plants		Disease reaction in field (No. of plants)			
		Monomorphic	Polymorphic	BW		ToLCV	
				R	S	R	S
F_2-06	50	46	04	30	20	02	28
F_2-18	50	32	18	28	22	04	24
F_2-20	50	50	00	22	28	02	20
F_2-33	50	06	44	24	26	04	20
	Total	134	66	104	96	12	92



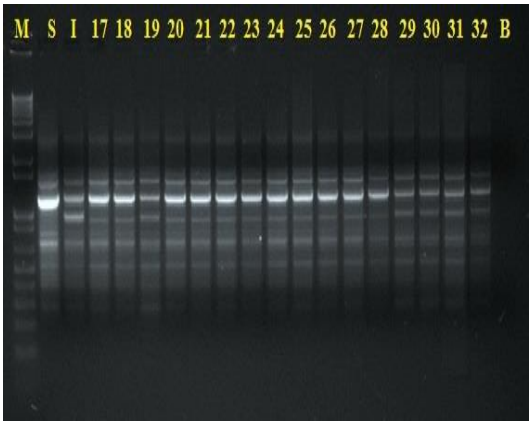
F_2-18-1 to 16



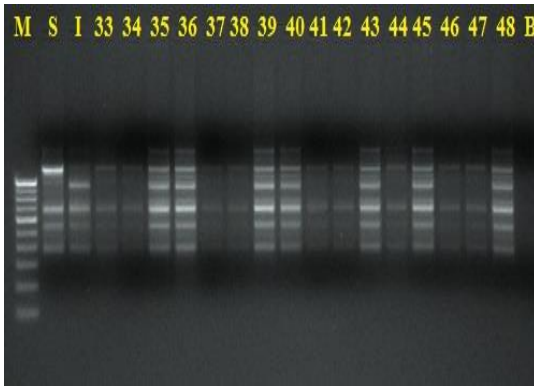
F_2-33-1 to 16



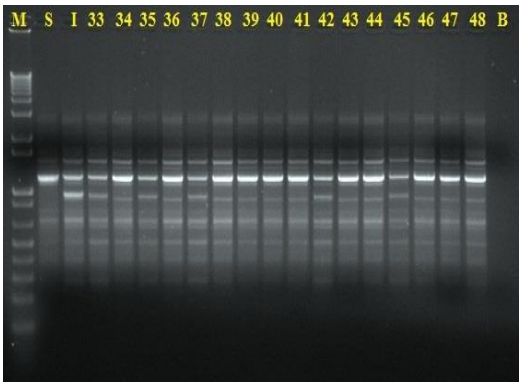
F_2-18-17 to 32



F_2-33-17 to 32



F_2-18-33 to 48



F_2-33-33 to 48

M- Marker (1 or 3 Kb), S- Sakthi, I- IIHR 2196, B- Blank

Plate 11: ISSR amplification pattern of F_3 population with primer HB 12

population and compared with the disease reaction. The details of polymorphic band observed in the F_3 population are provided in Table 16. The marker segregation and field reaction observed in F_3 population is provided in annexure III.

4.4.3 SSR assay on F_3 population

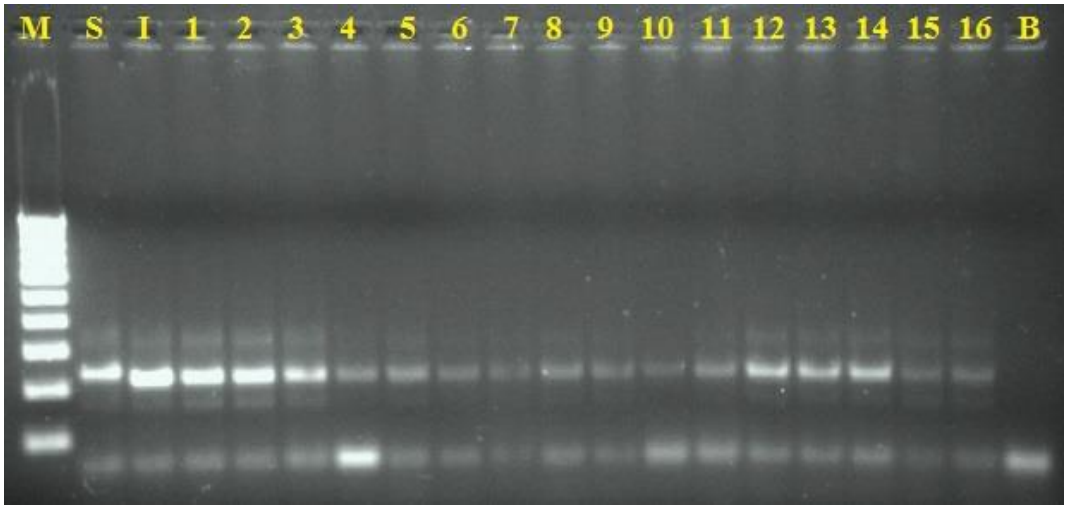
SSR analysis was carried out for the two hundred plants with the selected primers. Out of fourteen primers evaluated with the parents, three were selected for screening the F_3 population. These included SSR KAU 11, SSR 450 and LEaat 16.

Amplification pattern of SSR KAU 11 in F_3 population

The SSR profile generated by this primer displayed monomorphic banding pattern in all F_3 progenies (Plate 12a). The size of monomorphic band was 270 bp. The details of amplification observed in F_3 population are provided in Table 19. The marker segregation scored against disease reaction observed in F_3 population is summarised in Table 17. This primer did not show clear polymorphism with respect to bacterial wilt and ToLCV.

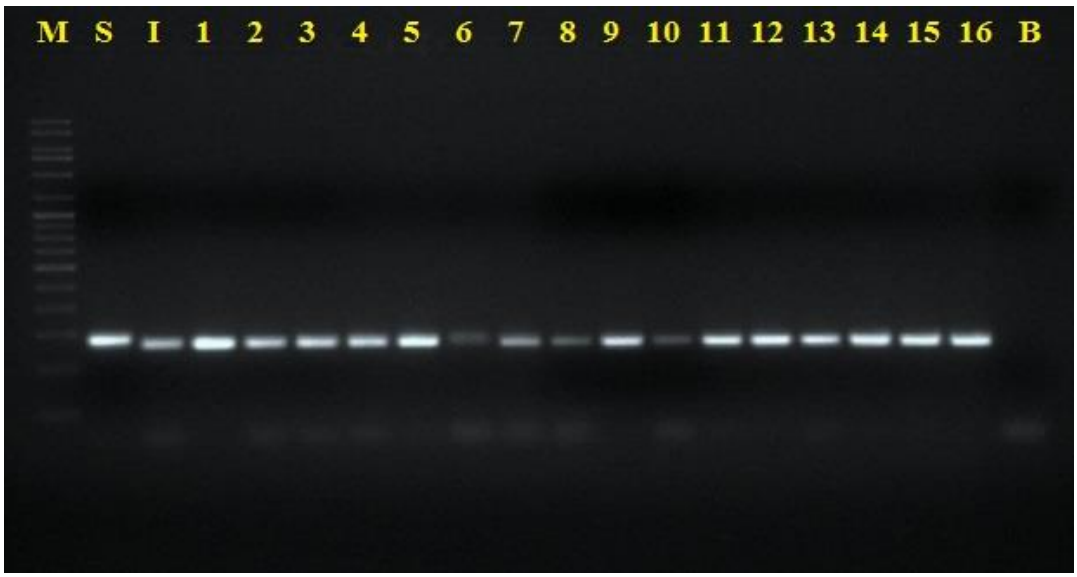
Amplification pattern of SSR 450 in F_3 population

This primer generated one band in both Sakthi and IIHR 2196 with slight polymorphism. The molecular weight of the amplicons was 280-300 bp. This primer produced one clear amplicon which shown the monomorphic banding patterned in F_3 population (Plate 12b) includes resistance and susceptible plants. The details of polymorphic and monomorphic band present in F_3 population are provided in Table 18. The size of polymorphic amplicon was observed 280 bp. The SSR 450 did not show polymorphism with respect to bacterial wilt and ToLCV.



M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, 1 to 16- Plant F2-06-1 to 16, B- Blank

Plate 12a: SSR amplification pattern of F_3 population with primer SSRKAU 11



M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, 1 to 16- Plant F2-06-1 to 16, B- Blank

Plate 12b: SSR amplification pattern of F_3 population with primer SSR 450

Table- 17 Amplification pattern for the primer SSRKAU 11 (Specific for Bw and ToLCV) in F_3 population

Population (Mother plants)	No. of F_3 progenies evaluated	No. of plants		Disease reaction in field (No. of plants)			
		Monomorphic	Polymorphic	BW		ToLCV	
				R	S	R	S
F_2-06	50	50	00	30	20	02	28
F_2-18	50	50	00	28	22	04	24
F_2-20	50	50	00	22	28	02	20
F_2-33	50	50	00	24	26	04	20
	Total	200	00	104	96	12	92

Table- 18 Amplification pattern for the primer SSR 450 (Specific for BW) in F_3 population

Population (Mother plants)	No. of F_3 progenies evaluated	No. of plants		Disease reaction in field (No. of plants)			
		Monomorphic	Polymorphic	BW		ToLCV	
				R	S	R	S
F_2-06	50	50	00	30	20	02	28
F_2-18	50	50	00	28	22	04	24
F_2-20	50	50	00	22	28	02	20
F_2-33	50	50	00	24	26	04	20
	Total	200	00	104	96	12	92

Table- 19 Amplification pattern for the primer LEaat 16 (Specific for BW) in F_3 population

Population (Mother plants)	No. of F_3 progenies evaluated	No. of plants		Disease reaction in field (No. of plants)			
		Monomorphic	Polymorphic	BW		ToLCV	
				R	S	R	S
F_2-06	50	11	39	30	20	02	28
F_2-18	50	15	35	28	22	04	24
F_2-20	50	27	23	22	28	02	20
F_2-33	50	17	33	24	26	04	20
	Total	70	130	104	96	12	92

Amplification pattern of LEaat 16 in F_3 population

This primer gave 270 bp size band in Sakthi and no amplification was observed in IIHR 2196. The details of amplification in F_3 population are provided in Plate 13 and 14. The detail of polymorphic and monomorphic band present in F_3 population is provided in Table 19. The marker segregation scored against disease reaction observed in F_3 population and details provided in annexure III.

4.4.4 SCAR assay on F_3 population

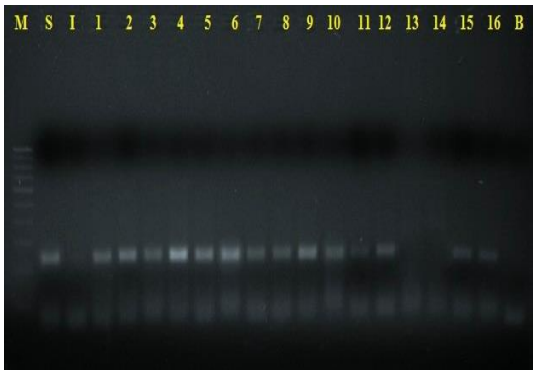
The SCAR primers selected for evaluation of F_3 population included $SCAR_{AAG/CAT}$ and Ualty 16. DNA isolated from the 200 F_3 progenies were amplified at specific condition with these two primers.

Amplification pattern of $TSCAR_{AAG/CAT}$ in F_3 population

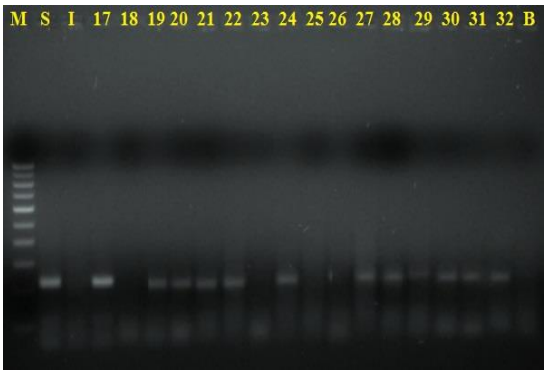
This primer produced two amplicons in Sakthi and one in IIHR 2196. The size of polymorphic amplicon was 500 bp. The details of amplification in F_3 population are provided in Plate 15 and 16. The details of polymorphism observed in F_3 population are provided in Table 20. The marker segregation scored against disease reaction observed in F_3 population and details provided in annexure III.

Amplification pattern of Ualty 16 in F_3 population

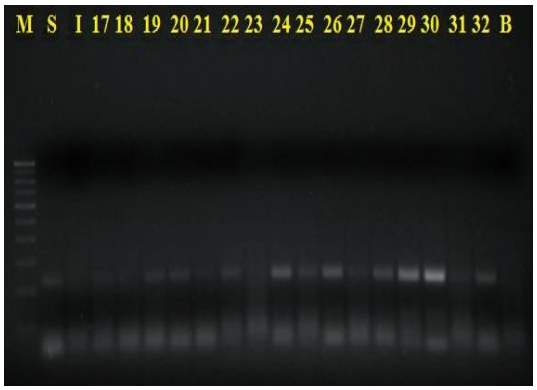
This primer when amplified with parents Sakthi and IIHR 2196 generated one band in IIHR 2196 which is resistant to ToLCV. In F_3 population 148 progenies gave monomorphic and 52 polymorphic amplicons (Plate 17 and 18). The molecular size of polymorphic amplicon was about 500 bp. The SCAR profile generated by this primer displayed monomorphic and polymorphic banding pattern in F_3 progenies (Table 21). The marker segregation scored against disease reaction observed in F_3 population is summarised in Table 18 and details provided in annexure III.



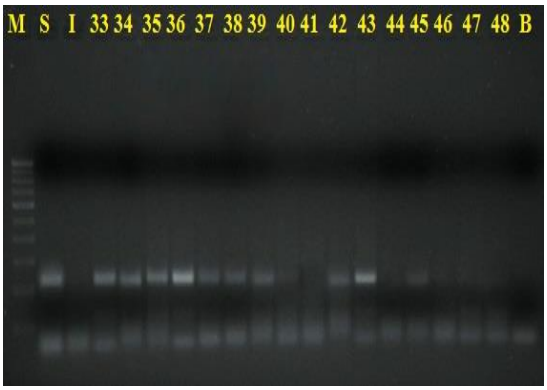
F_2-06-1 to 16



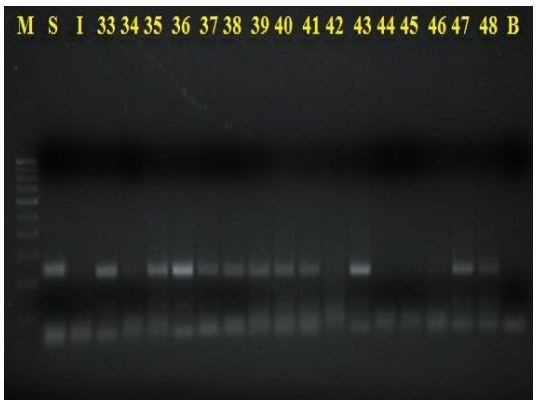
F_2-18-17 to 32



F_2-06-17 to 32

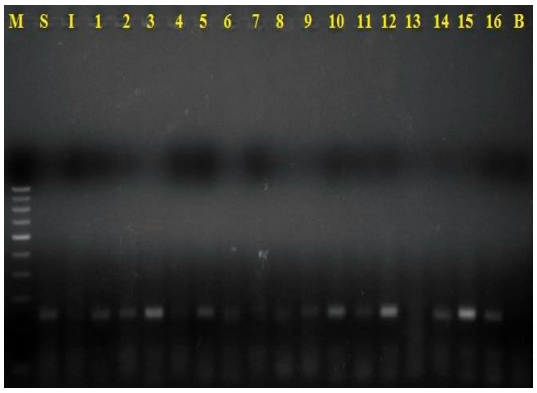


F_2-18-33 to 48

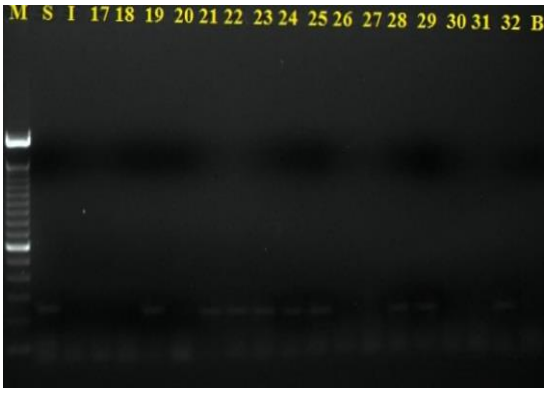


F_2-06-33 to 48

F_2-20-1 to 16



F_2-18-1 to 16

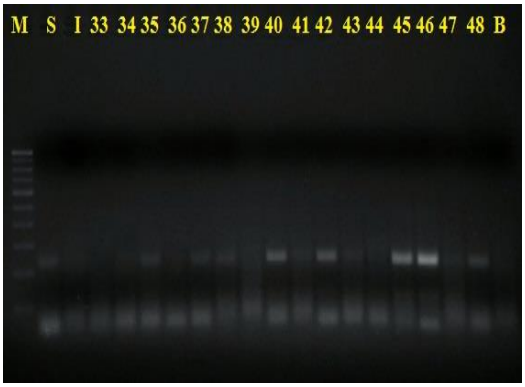


F_2-20-17 to 32

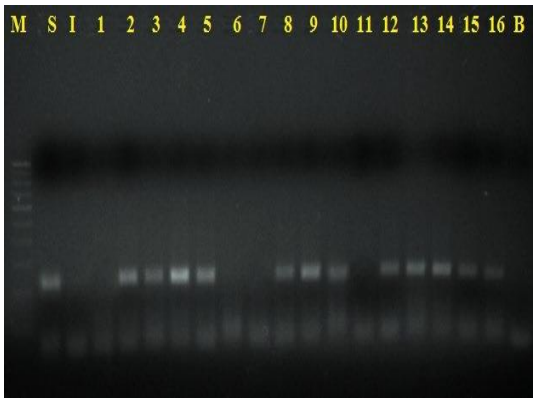
M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, B- Blank

Plate 13: SSR amplification pattern of F_3 population with primer LEaat 16

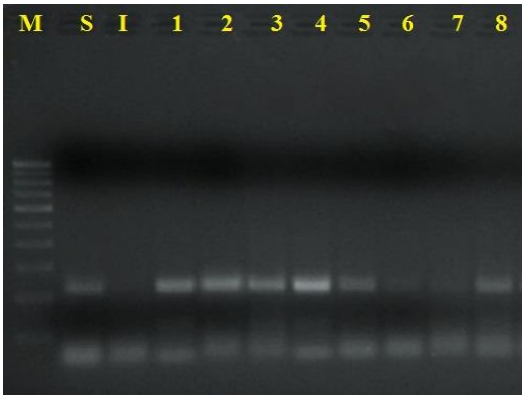
F_2-20-33 to 48



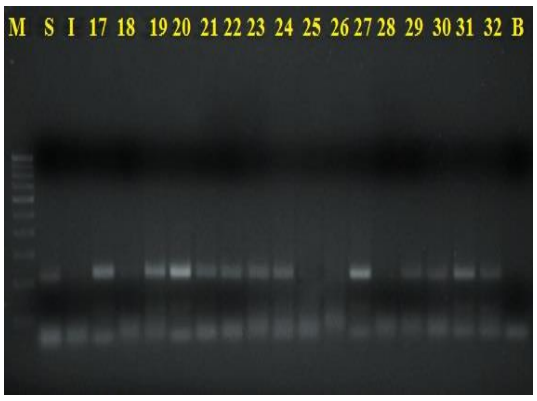
F_2-33-33 to 48



F_2-33-1 to 16



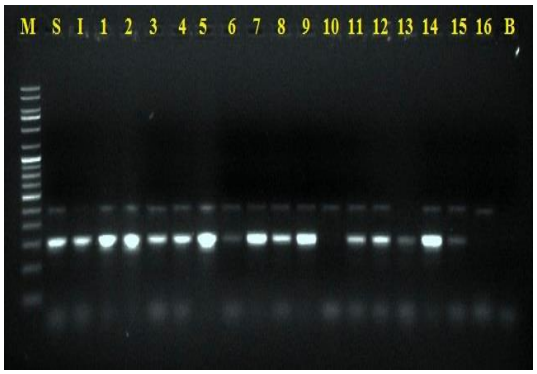
M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, B- Blank, 1- F_2-06-49, 2- F_2-06-50, 3- F_2-18-49, 4- F_2-18-50, 5- F_2-20-49, 6- F_2-20-50, 7- F_2-33-49, 8- F_2-33-50



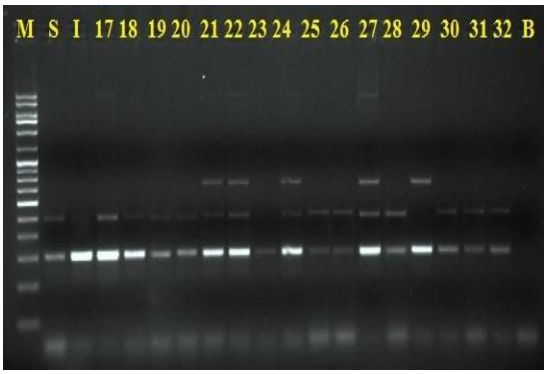
F_2-33-17 to 32

M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, B- Blank

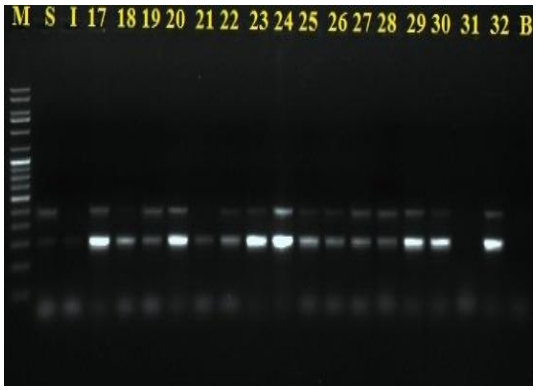
Plate 14: SSR amplification pattern of F_3 population with primer LEaat 16



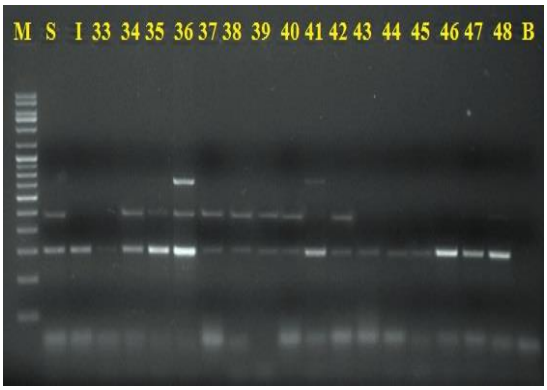
F_2-06-1 to 16



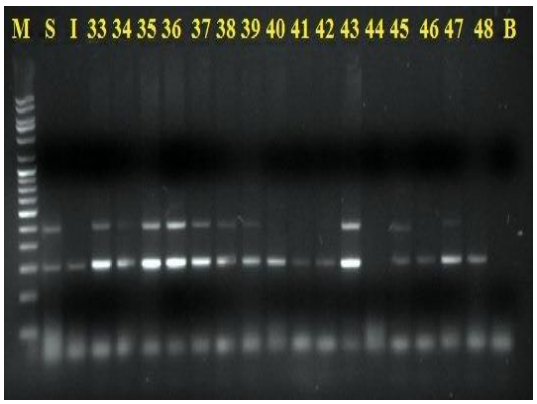
F_2-18-17 to 32



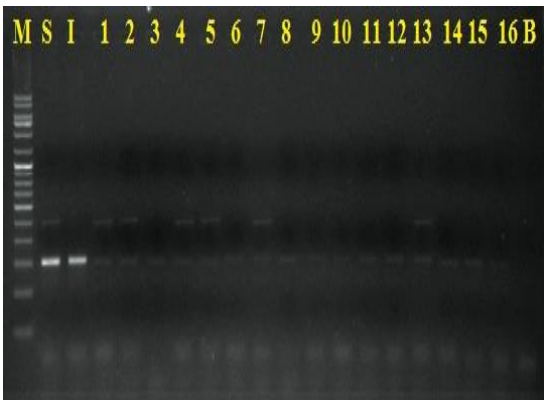
F_2-06-17 to 32



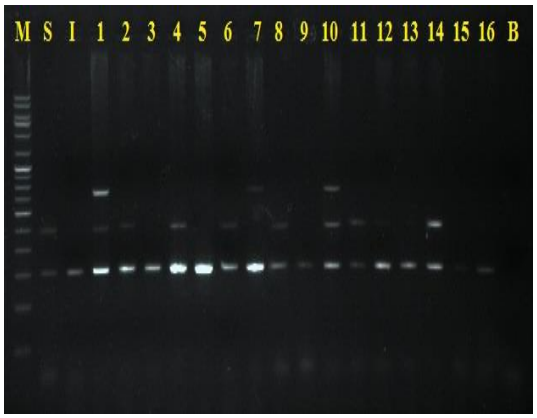
F_2-18-33 to 48



F_2-06-33 to 48



F_2-20-1 to 16

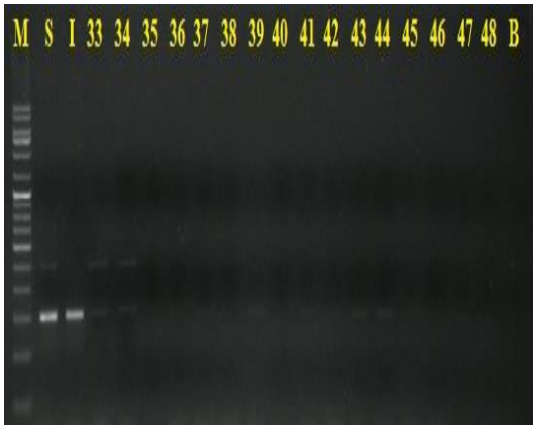


F_2-18-1 to 16

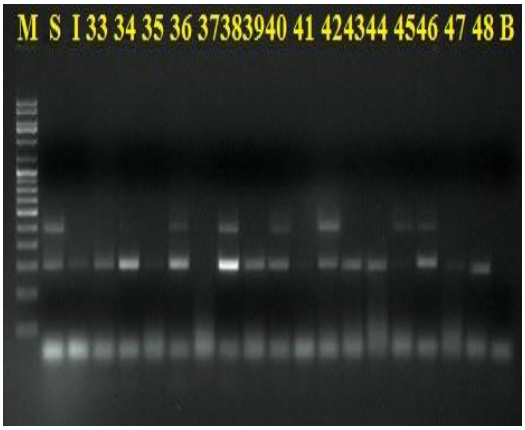
F_2-20-17 to 32

M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, B- Blank

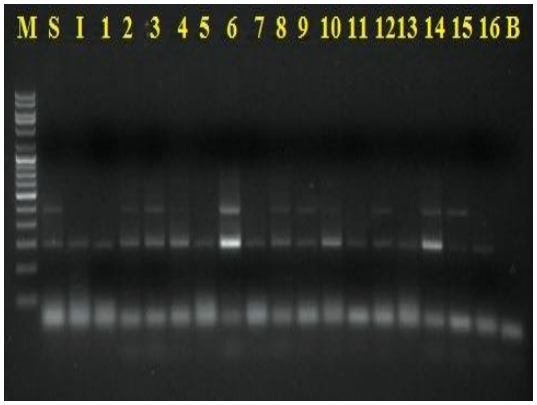
Plate 15: SCAR amplification pattern of F_3 population with primer $TSCAR_{AAG/CAT}$



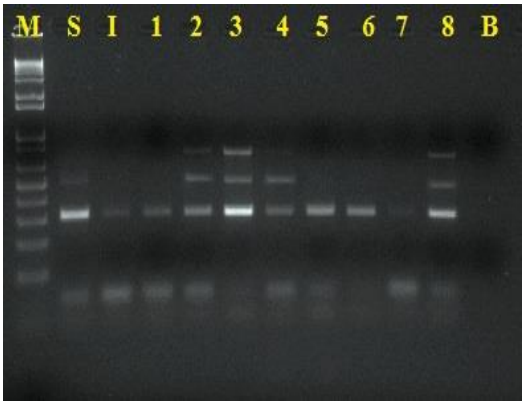
F_2-20-33 to 48



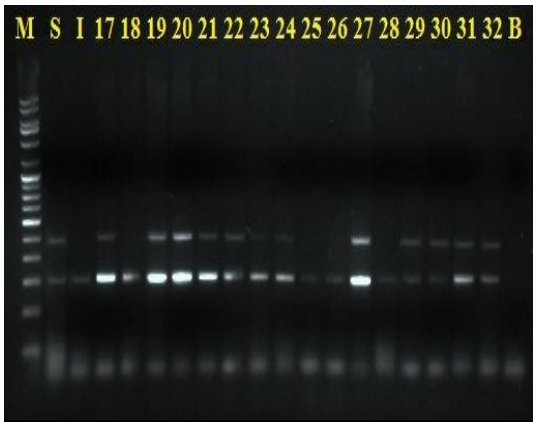
F_2-33-33 to 48



F_2-33-1 to 16



M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, B- Blank, 1- F_2-06-49, 2- F_2-06-50, 3- F_2-18-49, 4- F_2-18-50, 5- F_2-20-49, 6- F_2-20-50, 7- F_2-33-49, 8- F_2-33-50



F_2-33-17 to 32

M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, B- Blank

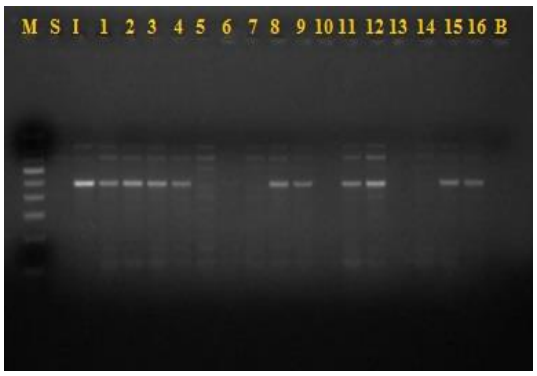
Plate 16: TSCAR amplification pattern of F_3 population with primer $SCAR_{AAG/CAT}$

Table- 20 Amplification pattern for the primer $SCAR_{AAG/CAT}$ (Specific for BW) in F_3 population

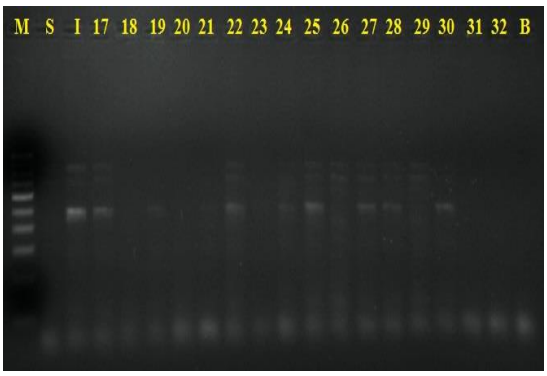
Population (Mother plants)	No. of F_3 progenies evaluated	No. of plants		Disease reaction in field (No. of plants)			
		Monomorphic	Polymorphic	BW		ToLCV	
				R	S	R	S
F_2-06	50	11	39	30	20	02	28
F_2-18	50	18	32	28	22	04	24
F_2-20	50	36	14	22	28	02	20
F_2-33	50	22	28	24	26	04	20
	Total	87	113	104	96	12	92

Table- 21 Amplification pattern for the primer Ualty 16 (Specific for ToLCV) in F_3 population

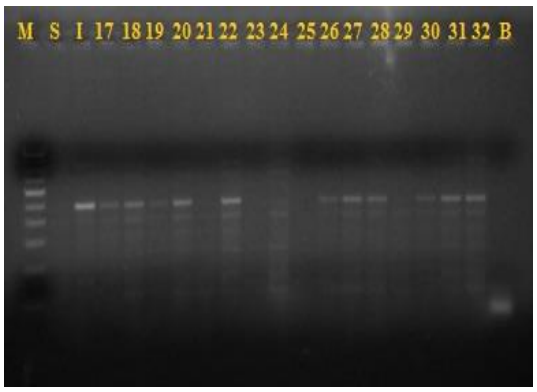
Population (Mother plants)	No. of F_3 progenies evaluated	No. of plants		Disease reaction in field (No. of plants)			
		Monomorphic	Polymorphic	BW		ToLCV	
				R	S	R	S
F_2-06	50	28	22	30	20	02	28
F_2-18	50	33	17	28	22	04	24
F_2-20	50	50	00	22	28	02	20
F_2-33	50	37	13	24	26	04	20
	Total	148	52	104	96	12	92



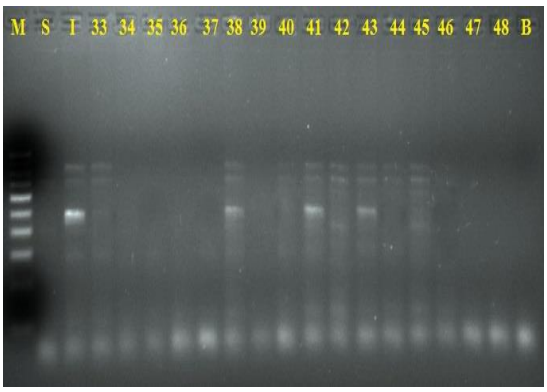
F_2-06-1 to 16



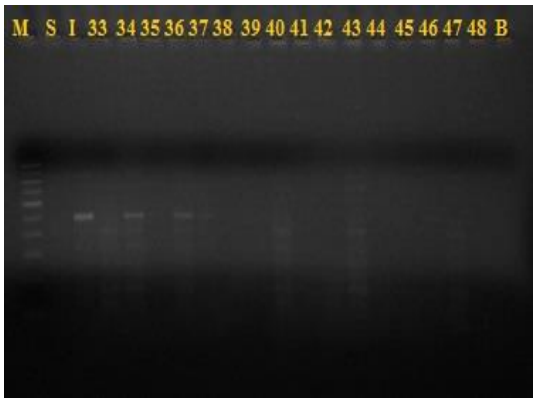
F_2-18-17 to 32



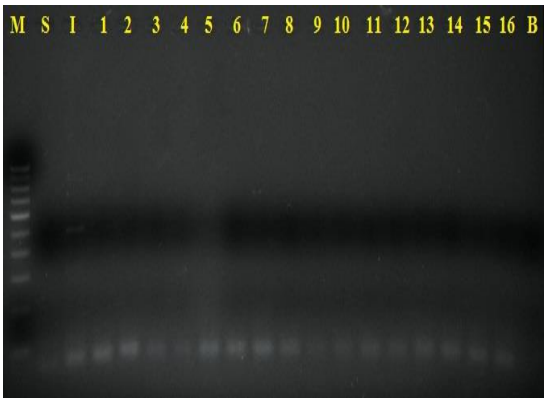
F_2-06-17 to 32



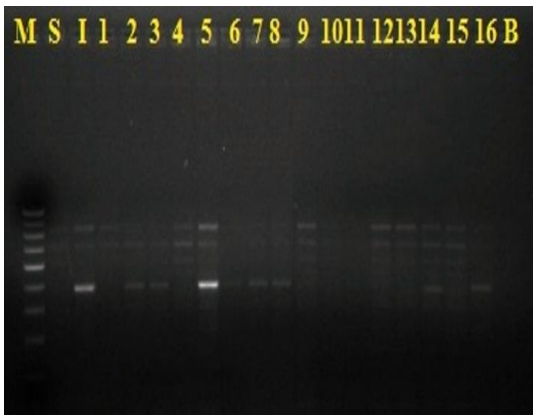
F_2-18-33 to 48



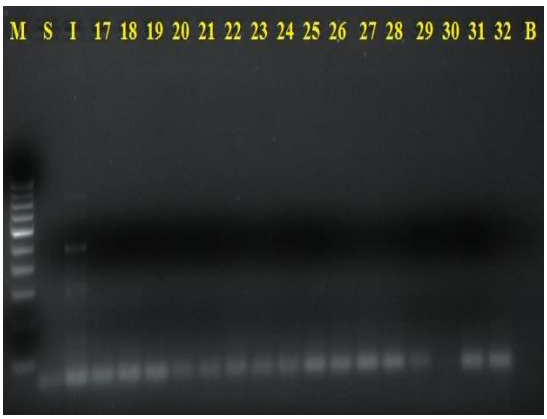
F_2-06-33 to 48



F_2-20-1 to 16



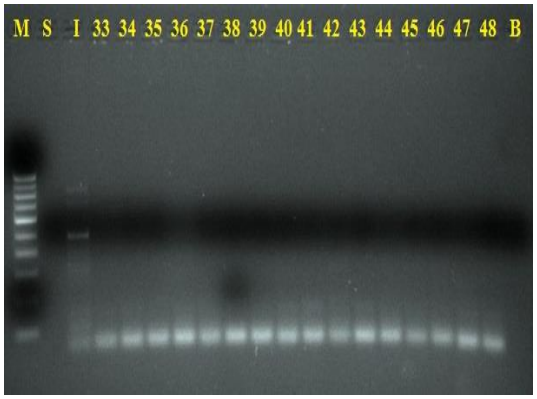
F_2-18-1 to 16



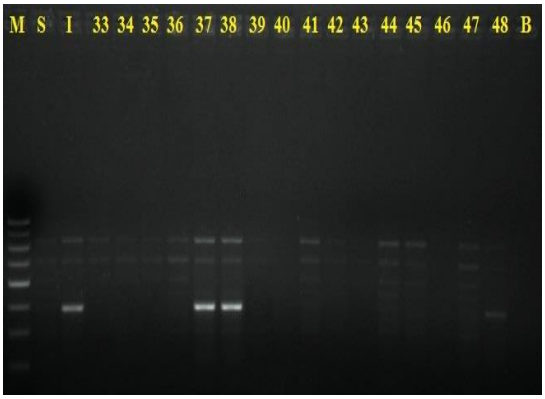
F_2-20-17 to 32

M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, B- Blank

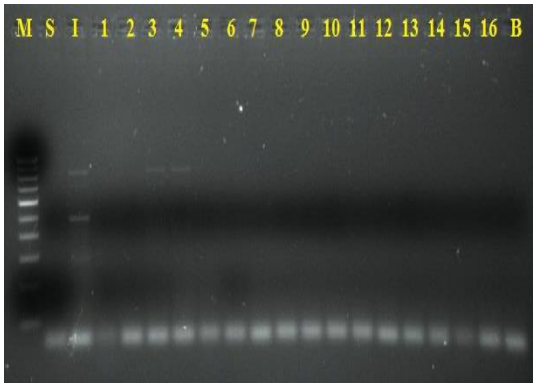
Plate 17: SCAR amplification pattern of F_3 population with primer Ualty 16



F_2-20-33 to 48

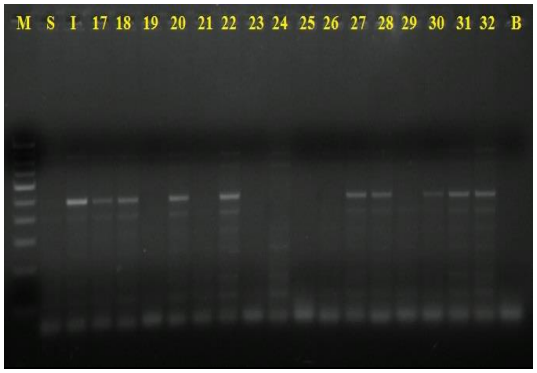


F_2-33-33 to 48



F_2-33-1 to 16

M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, B- Blank, 1- F_2-06-49, 2- F_2-06-50, 3- F_2-18-49, 4- F_2-18-50, 5- F_2-20-49, 6- F_2-20-50, 7- F_2-33-49, 8- F_2-33-50



F_2-33-17 to 32

M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, B- Blank

Plate 18: SCAR amplification pattern of F_3 population with primer Ualty 16

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 borne pathogen *Ralstonia solanacearum* (Yabuuchi *et al*., 1995) and the Tomato Leaf Curl Virus. Because of the devastating nature of these diseases, the productivity greatly depends on the field reaction and control measures adopted. Significant variation exists for the tolerance levels and defence mechanisms expressed by different tomato genotypes grown in the country. Tomato varieties have been identified at different research centres for tolerance/resistance to location specific diseases. However, genotypes with combined resistance and better agronomic traits are greatly warranted to improve tomato cultivation in problematic areas. This study is such an attempt to introgress ToLCV resistance and better agronomic traits of IIHR 2196 to the bacterial wilt resistance background of variety Sakthi through marker assisted selection.

The use of molecular markers to assist in separating bacterial wilt resistance from undesirable horticultural characteristics and to pyramid resistance characteristics from multiple sources, have been most demonstrated in breeding (Yang and Francis, 2007). Using different resistance sources and pathogen strains, several QTLs for resistance to bacterial wilt were identified on tomato chromosomes 3, 4, 6, 7, 8, 10 and12 (Mangin *et al*., 1999).

Traditional breeding has resulted in development of cultivars with reduced susceptibility, but no cultivar with complete resistance to ToLCV is available. In addition, the disease response of the resistant cultivars often varies from location to location, and it has been difficult to develop resistant cultivars with horticultural characteristics similar to those of susceptible ones (Scott, 2007).

Attempts on management of these diseases have not yielded so far any effective and efficient control strategy. Therefore the viable technology left to

combat the diseases is the use of resistant varieties. Resistance breeding taken up in Kerala Agricultural University has resulted in the development of three bacterial wilt resistant varieties *viz*., Sakthi, Mukthi, Anagha and one tolerant variety Vellayani Vijay. But these varieties are found susceptible to tomato leaf curl virus disease. Arka Ananya, IIHR 2195, IIHR 2196, IIHR 2197, IIHR 2198 and IIHR 2202 were highly resistant to ToLCV (Yadav, 2011). The resistance in IIHR 2195, IIHR 2196 and IIHR 2198 were earlier reported by Singh and Sadashiva (2007).

When sources of resistance to bacterial wilt and tomato leaf curl virus are available, these two characters can be combined in a single genotype with the help of marker assisted selection and this will be a turning point in tomato cultivation. Keeping this as the objective, the present investigation was undertaken.

Response of tomato F_3 population derived from the parents Sakthi (bacterial wilt resistant) and IIHR 2196 (ToLCV resistant) were evaluated in the field and the pattern of marker segregation was studied through molecular assay in order to validate the reported markers for marker assisted selection in the selected genotypes. The results obtained are discussed here.

5.1 Sources of resistance to bacterial wilt and ToLCV

In the present study, the genotype Sakthi was used as bacterial wilt resistance source and IIHR 2196 as a ToLCV resistance source. The resistance of Sakthi to bacterial wilt has been reported earlier by Sadhankumar (1995) and Karumannil *et al*. (2008). The resistance of IIHR 2196 to ToLCV has been earlier reported by Singh and Sadashiva (2007) and Yadav (2011). These two parents were used for developing combined disease resistance in a single genotype under the *SOL* Genome Project being implemented at Kerala Agricultural University. Four F_2 lines reported to be resistant to both ToLCV and bacterial wilt (Yadav, 2011) were selected for raising the F_3 population in the present investigation.

5.2 Combining ability, gene action and heterosis

In a heterosis breeding programme, selection of parents and mother plants are based on the information on gene action and knowledge of combining ability. In the present study, the survived F_3 plants were screened. The significant variance in Duncan test has indicated both additive and non-additive gene action for the control of biometrical characters.

5.2.1 Plant height

The plant height ranged from 32 to 62 an in the parent Sakthi and 52 to 72 an in IIHR 2196 (Fig. 1a). Analysis of variance indicated superiority of for F_2-18 (60.93) with respect to plant height. However, the F_2-33-37 (98 cm) plant was found tallest among the survived plants. Plant height is usually indicative of its vegetative vigour which influences the plant productivity (Patil, 2001).

5.2.2 Days to flowering

Number of days taken by a plant to put forth the first flower is generally indicative of its earliness. There was no significant variation observed in F_3 population with respect to days to flowering (Fig. 1b). However, F_2-33-12 plant was found early flowering. Heterosis for days to flowering in tomato has been reported by Dhaliwal *et al*. (2003).

5.2.3 Days to fruiting

Number of days taken by a plant to set the first fruit is generally indicative of its earliness. There was no significant variation observed in F_3 population studied (Fig. 1b). The line, F_2-33-12 was found early fruiting.

5.2.4 Number of fruits per plant

Lines from F_2-33 (92.00) were superior for number of fruits per plant as evidenced by Duncan test. Heterosis for number of fruits and variation in segregating population has been reported by Patil (2001). F_2-33-30, F_2-33-38, and

F_2-33-46 had shown heterosis for number of fruits per plant (92, 91 and 86). The graphical representation with respect to number of fruits per plant is given in Fig. 1c.

5.2.5 Yield per plant

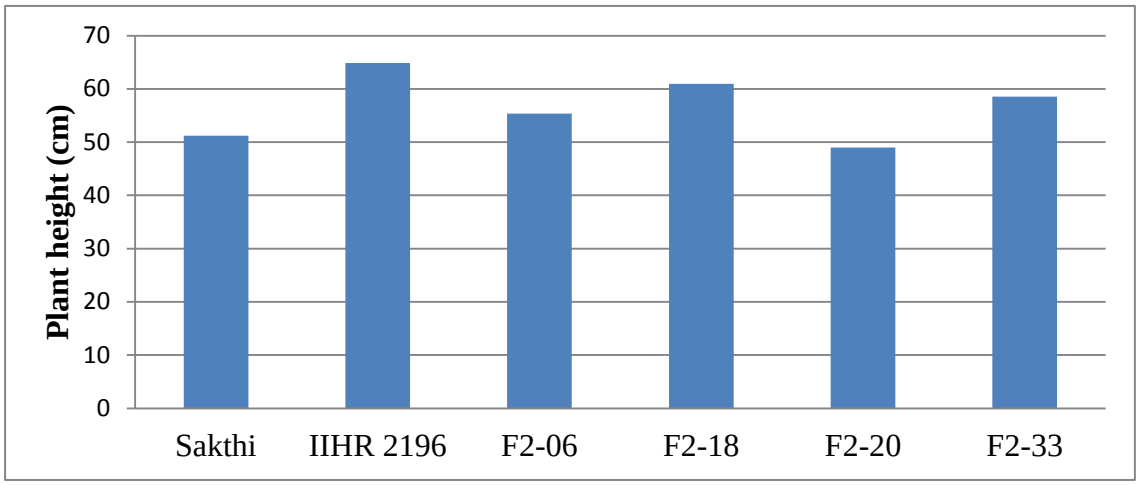
Lines from F_2-33 were also superior for yield per plant (1347.27 g) as evidenced by Duncan post hoc test. This group was also superior for number of fruits which would have contributed to the total yield (Fig. 2a). Patil (2001) has reported positive heterosis for this trait and good segregation, as expected was observed in the F_3 population.

5.2.6 Average fruit weight

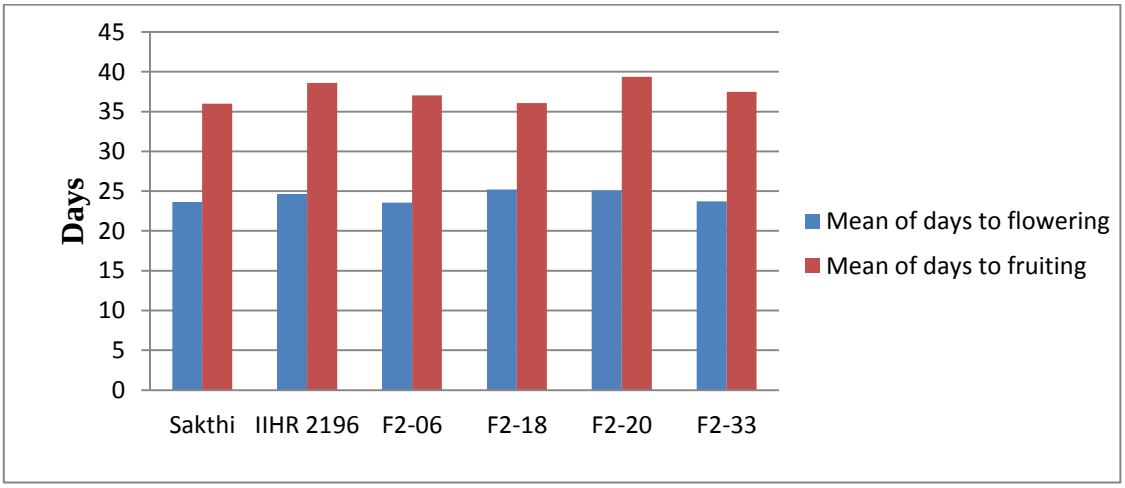
Analysis of variance indicated superiority for F_2-33 (30.36 g) with respect to average fruit weight also (Fig. 2b). The average fruit weight was highest in plant F_2-33-37 (3281 g). Heterosis for average fruit weight was reported by Bhushana (2000).

5.3 Field reaction of F_3 population for bacterial wilt and ToLCV incidence

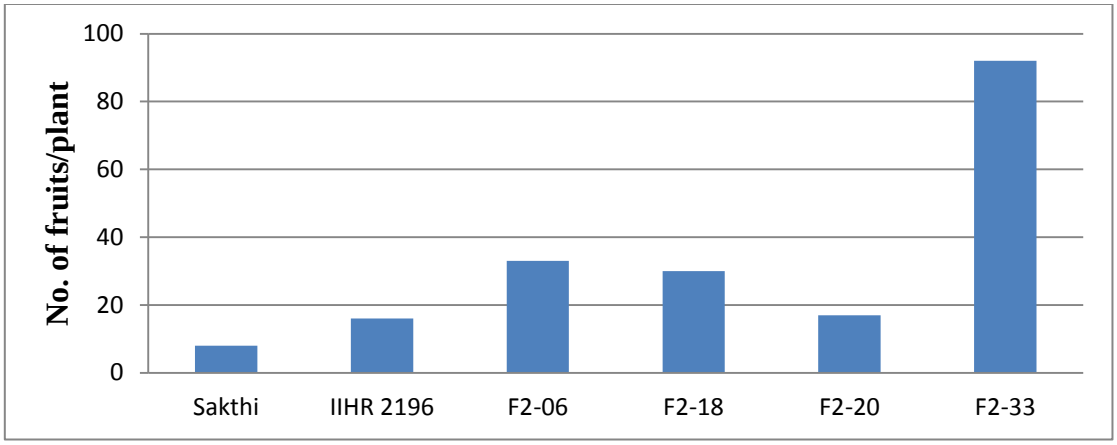
Two hundred F_3 seedlings raised out of four F_2 lines were transplanted to field, 30 days after germination during October 2013 to January 2014. Wilting started one week after transplanting, as leaf drooping, followed by complete wilting and death of the plants. Bacterial wilt incidence was confirmed by ooze test. Maximum wilt incidence was observed between 30 to 45 days after transplanting. However, no wilting was observed 60 days after transplanting. Younger seedling succumbed to wilt more rapidly since seedlings had thinner cortical calls compared to older plants that make the entry of pathogen easier. Celine (1981) also highlighted that wilting was more in juvenile stage as compared to mature plants.



a. Plant height

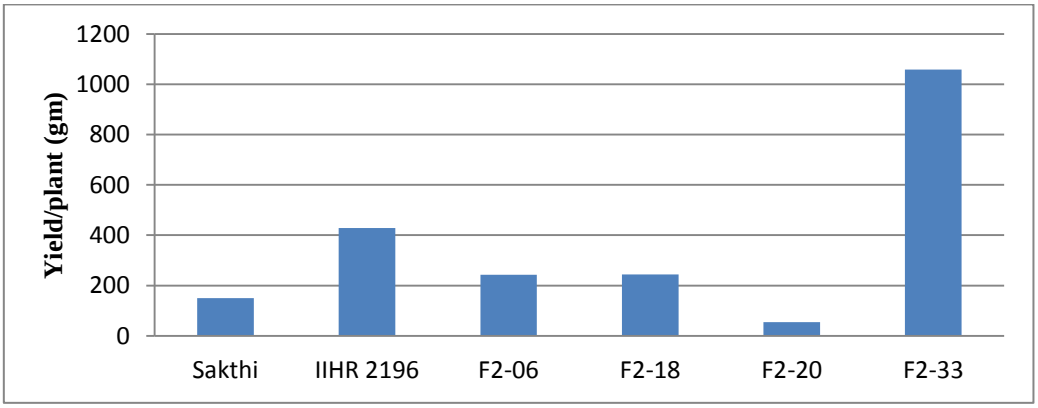


b. Days to flowering and fruiting

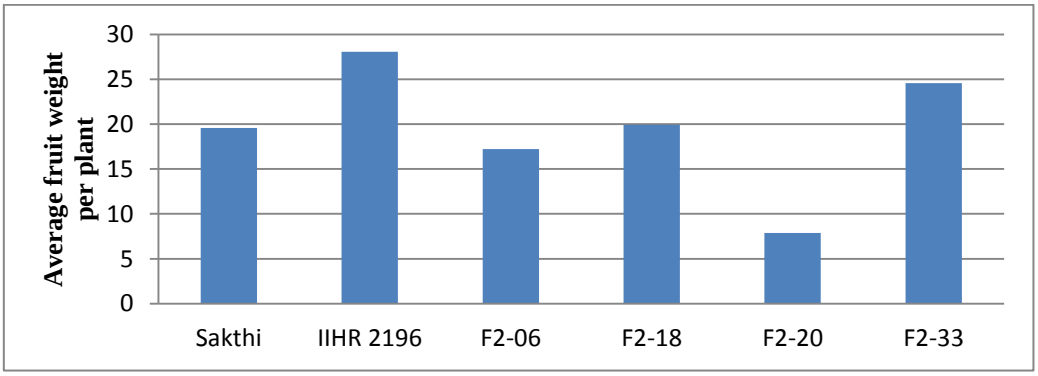


c. Number of fruits per plant

Fig. 1: Mean performance of parental line (Sakthi and IIHR 2196) and F_2 progenies with respect to plant height, days to flowering and fruiting and no. of fruits per plant



a. Yield per plant



b. Average fruit weight

Fig. 2: Mean performance of parental line (Sakthi and IIHR 2196) and F_2 progenies with respect to yield per plant and average fruit weight

F_3 population derived from the four F_2 plants had shown different disease reactions (Fig. 3). Survival rate was observed to be more in plants derived from F_2-06 (60 %) and low in F_2-20 (44 %).

Tomato leaf curl incidence started 25 days after transplanting, as very mild curling which advanced to puckering and severe curling. The severity of ToLCV has increased day by day after one month transplanting. The minimum disease incidence was observed one month after transplanting and maximum disease incidence was observed from two months after transplanting. For calculation of coefficient of infection for ToLCV, the plants were scored two months after transplanting.

In F_3 population, only few plants had shown resistance to ToLCV. The per cent of disease incidence has differed from that of mother plants. In F_2-18 and F_2-33 progenies, four plants were observed resistant to ToLCV and in F_2-06 and F_2-20, two plants were resistant. In F_3 population some plants failed to produce the fruits due to high severity of ToLCV. The detail of disease incidence is provided in Table 8 under the section 4.1.2.

5.4.1 Isolation of genomic DNA

The DNA was isolated from young leaves of tomato plants including the parents and F_3 progenies. The protocol suggested by Roger and Bendich (1994) has yielded good quality DNA. The electrophoresis of DNA has shown distinct bands without shearing. The genomic DNA isolated through the CTAB method was pure with slight RNA contamination. RNase treatment given during DNA isolation was found effective than RNase treatment given after the DNA isolation. The homogenisation and uniform grinding of tender leaves were essential during DNA isolation. Excess liquid nitrogen was used for the homogenisation of leaf tissue. Liquid nitrogen helps in maintaining the frozen tissue, preventing nucleic acid degradation and effect of secondary metabolites. The problem of polyphenol was overcome by the addition of β-mercaptoethanol, poly vinyl and

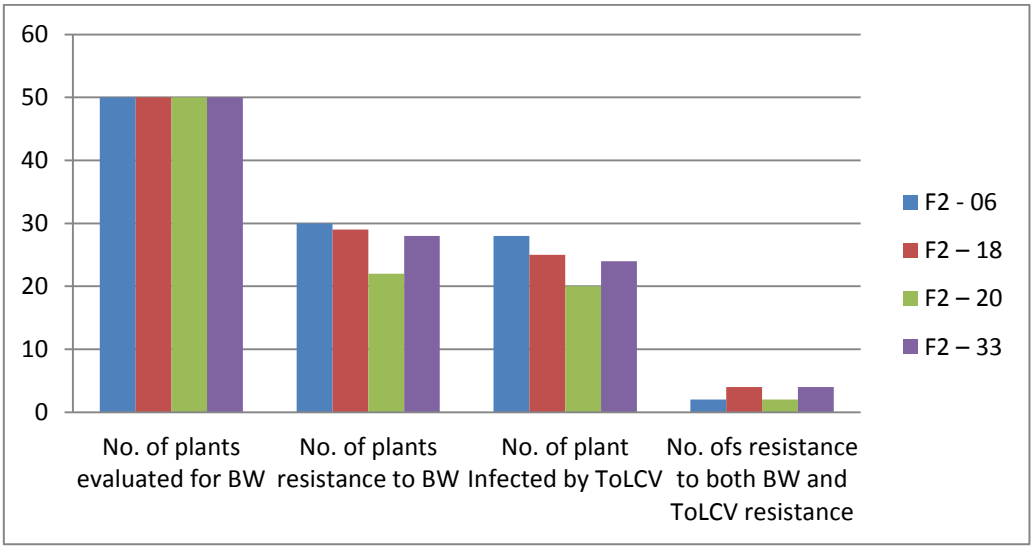


Fig. 3: Disease reaction variation in different F_2 parental lines for BW and ToLCV resistance

pyrrolidone (PVP) along with extraction buffer. The PVP removes polyphenols and inhibits co-precipitation of polysaccharide which resulted in good quality DNA (Hernandez and Oyaszum, 2006).

The detergent, CTAB, helps to release nucleic acids into the buffer after disruption of the cell membrane. The released DNA is protected from the action of DNase enzyme with presence of EDTA in the extraction buffer. It is a chelating agent, which efficiently blocks Mg^{2+} ions, which is the major cofactor for DNase enzyme. The DNA isolated by CTAB method was freed from chlorophyll by using the Chloroform: Isoamyl alcohol which aids in the separation of organic mixture and aqueous phase of the DNA isolation. EDTA was also a major component of TE buffer in which DNA is dissolved and stored.

5.4.2 Screening with parents Sakthi and IIHR 2196 with reported primers

Parents were screened with the previously reported ISSR, SSR and SCAR primers. The primers included fourteen ISSR, fourteen SSR and eight SCAR primers. Out of these few primers gave the polymorphisms in parents including one ISSR (HB 12), three SSR (SSR KAU 11, SSR 450 and LEaat 16) and two SCAR ($TSCAR_{AAG/CAT}$ and Ualty 16).

5.5 Screening of F_3 population with molecular marker for bacterial wilt and ToLCV

Different primers reported for bacterial wilt and ToLCV in tomato were first validated on the parents and the selected ones used for screening the F_3 DNA. All the 200 F_3 progenies were screened with the selected primers belonging to ISSR, SSR and SCAR. Trait specific molecular markers will be the ideal tool for marker assisted selection. Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based markers.

5.5.2 ISSR assay on F_3 population

Five reported primers were selected for screening the parents based on the previous studies conducted on isolation and characterization of genes encoding for bacterial wilt and ToLCV disease resistance in tomato under the Sol Genome Project. The primer ISSR KAU 7 showed trait specific polymorphism at two regions (0.27 Kb and 1.2 KB) and distinct band observed in the resistant genotypes H8878, EC520061 and Anagha and absent in all susceptible accessions reported by Nazeem *et al*. (2010). HB 12 marker was selected for screening the F_3 population based on the polymorphism pattern given in the parent. DNA of Sakthi amplified with the primer HB 12 gave six amplicons and IIHR 2196 it gave seven amplicons. From those seven bands, one was polymorphic in ToLCV resistant IIHR 2196. The details of marker HB 12 segregation scored against disease reaction in F_3 population given in Table 22.

The marker system called ISSR (Inter Simple Sequence Repeats) is a PCR based method that assesses variation in the numerous microsatellite regions dispersed throughout the genome. In this technique reported by Zietkiewicz *et al*. (1994), primers based on microsatellites are utilized to amplify inter simple sequence repeat sequences in the DNA. When the primer successfully locates two microsatellite regions within an amplifiable distance away on the two strands of the template DNA, the PCR reaction generates a band of a particular molecular weight for that locus representing the intervening stretch of DNA between the microsatellites. The method uses a single oligonucleotide primer composed of 4 to 10 tri or di nucleotide repeats and ending with 3'- or 5'- anchor sequence.

The ISSR marker requires small amounts of DNA and does not require information on DNA sequence. ISSR targets the highly variable microsatellite regions of the nuclear genome providing a large number of polymorphic fragments (Gupta *et al.,* 1994). Therefore this technique has been used extensively to evaluate genetic variation both within and between plant populations in angiosperms and gymnosperms (Osborn *et al*., 2005).

Table- 22 The markers segregation scored against disease reaction in F_3 population

Sl. No.	Marker system	Primers	No. of plant analysed			Polymorphism of markers for disease reaction				Field reaction			
			Through Markers	In Field									
			Segregation	BW	ToLCV	BW		ToLCV		BW		ToLCV	
						R	S	R	S	R	S	R	S
01	**ISSR**	HB 12	200	200	104	-	-	66	134	104	96	12	92
02	**SSR**	SSR KAU 11	200	200	104	-	-	-	-	104	96	12	92
03	**SSR**	SSR 450	200	200	104	-	-	-	-	104	96	12	92
04	**SSR**	LEaat 16	200	200	104	130	70	-	-	104	96	12	92
05	**SCAR**	$TSCAR_{AAG/CAT}$	200	200	104	113	87	-	-	104	96	12	92
06	**SCAR**	Ualty 16	200	200	104	-	-	52	148	104	96	12	92

The ISSR markers are found useful in the marker assisted selection and in understanding the evolutionary relationships of various crops such as apple (Goulao and Oliveira, 2001), tomato (Terzopoulos and Bebeli, 2008) and *Auricularia auricula* (Li *et al*., 2008).

5.5.3 SSR assay on F_3 population

SSR (Simple sequence repeat) is the most reliable molecular marker system in molecular breeding. The results in many studies show that SSR markers are popularly used in genetic analysis of cultivars (Rajput *et al*., 2006).

In the present study, fourteen SSR primers were selected for screening the parents. Out of these, three primers gave polymorphic amplicons in the parents and these include SSRKAU 11, SSR 450 and LEaat 16. These were further used for evaluating the F_3 population.

Amplification pattern of SSRKAU 11 and SSR 450 in F_3 population

The data in the present study indicated that these primers have not shown any polymorphic amplicons in F_3 population. These two primers were not useful for bacterial wilt and ToLCV in F_3 population. Several QTLs for resistance to bacterial wilt were identified on tomato chromosomes 3, 4, 6, 7, 8, 10 and 12 (Thoquet *et al*., 1996; Mangin *et al*., 1999). It is understood that more genetic study is needed for understanding the molecular marker with respect to bacterial wilt and ToLCV. The details of markers SSRKAU 11 and SSR 450 segregation scored against disease reaction in F_3 population given in Table 22.

Amplification pattern of LEaat 16 in F_3 population

This primer gave the polymorphic amplicons in F_3 population. The data in the present study indicated that this primer has shown polymorphic amplicons in Sakthi genotype which is resistant to bacterial wilt. When morphological and molecular data were analysed, it had shown correlation with bacterial wilt

resistance. SSR 450, SSR 306, SSR LEaat 15 and LEaat 16 produced distinct polymorphism for bacterial wilt reported by Nazeem *et al*. (2010). The details of marker LEaat 16 segregation scored against disease reaction in F_3 population given in Table 22.

5.5.4 SCAR assay on F_3 population

This technique can be used to develop markers by using sequence specific PCR primers derived from RAPD, ISSR and AFLP fragments. The SCAR (Sequence Characterized Amplified Region) markers are designed based upon the specific nucleotide sequence and specific co-dominant PCR based marker. It is found stable, easy to use and inexpensive for single locus assay in different crops. Thus, the interpretation of the result is far straight forward as validation can be based on the presence and absence of a specific DNA fragment in a given population.

In this study, we have used eight specific primers which are specific for bacterial wilt and ToLCV. Out of eight primers two primers were selected for screening of F_3 population includes $TSCAR_{AAG/CAT}$ and Ualty 16.

Amplification pattern of $TSCAR_{AAG/CAT}$ in F_3 population

The data in the present study indicated that this primer had shown polymorphic amplicons with respect to bacterial wilt. When morphological and molecular data were analysed, it gave positive correlation with bacterial wilt resistance.

The bacterial wilt resistance is reported to be controlled by two complementary, co-dominant genes located on tomato chromosome 6. Two dominant SCAR markers, $TSCAR_{AAT/CGA}$ and $TSCAR_{AAG/CAT}$, were reported to be located 4.6 cM and 8.4 cM respectively, from a resistance gene *TRSR-1* and have been suggested useful for breeding for bacterial wilt resistance breeding via marker assisted selection (Foolad and Panthee, 2012).

Resistance breeding for bacterial diseases has been challenging in tomato. While some resistance genes have been identified and molecular markers associated with them reported, MAS is far from a routine application for improving bacterial disease resistance in tomato. It is either because of the complexity of pathogen, as is the case with bacterial spot, or the complexity of genetic resistance, as is the case with bacterial wilt and bacterial spot. However, in cases where the genetics of host resistance and pathogen are simple, as is the case with bacterial speck, MAS is being employed routinely. $TSCAR_{AAG/CAT}$ showed the good correlation with respect to bacterial wilt and it is good marker for developed bacterial wilt disease resistance through marker assisted selection.

Amplification pattern of Ualty 16 in F_3 population

This primer has shown the band in IIHR 2196 which is resistant to ToLCV. Four resistance loci, *Ty-1*, *Ty-2, Ty-3* and *Ty-4*, have been identified and mapped to tomato chromosomes 6, 11, 6 and 3, respectively (Ji *et al*., 2009b). These genes present in different chromosome are the constraint for developing specific marker for ToLCV disease. Several QTLs conferring resistance to ToLCV have also been identified (Agrama and Scott, 2006). Although these markers are reported, the lack of consistent genetic markers associated with ToLCV resistance has hindered the utility of MAS for this trait. In addition, since ToLCV is considered as a dangerous pathogen, screening germplasm for resistance as well as validation of any genetic marker has been challenging.

If root genotype available includes all the genes which are governing the ToLCV resistance in plant then it will be easy to develop a few specific markers for ToLCV in tomato.

In F_3 population (200 plants), 12 plants showed combined resistance to bacterial wilt and ToLCV and F_3 lines of the F_2-33 showed good yield parameters. ISSR marker (HB 12) and SCAR (Ualty 16) were found to segregate along with ToLCV resistance and SSR marker (LEaat 16) and SCAR ($TSCAR_{AAG/CAT}$) were found to segregate along with bacterial wilt resistance in F_3 population. Twelve

plants with combined resistance were screened out for further evaluation. Most of the plants with combined resistance were not good yielders, but four F_3 lines recorded good yield (2575 to 3281 g/plant).

The selected F_3 plants with combined resistance have to be forwarded for further evaluation (F_4, F_5….). Further confirmation of ISSR HB 12 marker is required for ToLCV resistance. LEaat 16 and $TSCAR_{AAG/CAT}$ markers can be recommended for bacterial wilt resistance and SCAR marker Ualty 16 can be recommended for ToLCV resistance in tomato through marker assisted selection. Segregation of these markers has to be evaluated on F_4 population.

Summary

SUMMARY

The investigations on "Integration of combined disease resistance for bacterial wilt and ToLCV in tomato (*Solanum lycopersicum* L.) through marker assisted selection" were carried out at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period from January 2013 to January 2014. The objective of the study was to investigate the inheritance pattern of the molecular markers identified and integrate combined disease resistance to bacterial wilt and ToLCV in tomato through marker assisted selection. Two tomato genotypes Sakthi (Bacterial wilt resistant) and IIHR 2196 (ToLCV resistant) were used for developing combined disease resistance. In the present study, F_3 population was screened for bacterial wilt and ToLCV at field level and using molecular markers.

The salient findings of the study are as follows:

1. Bacterial wilt resistant genotype Sakthi and ToLCV resistant genotype IIHR 2196 were raised in net house for extraction of genomic DNA. The protocol suggested by Roger and Bendich (1994) during DNA isolation RNase treatment was found suitable to yield good quality DNA.

2. Parental DNA (Sakthi and IIHR 2196) was used for screening the earlier reported primers which are specific for bacterial wilt and ToLCV resistance.

3. Fourteen ISSR, 14 SSR and 8 SCAR primers were screened for polymorphism in parents Sakthi and IIHR 2196. Out of these, only six primers (ISSR HB 12, SSRKAU 11, SSR 450, SSR LEaat 16, $TSCAR_{AAG/CAT}$ and Ualty 16) gave polymorphism in the parents.

4. Seeds collected from F_2 plants which were resistant to bacterial wilt and ToLCV (F_2-06, F_2-18, F_2-20 and F_2-33) were used for raising the F_3

population during October 2013 to January 2014. Sixty seeds were germinated from each of the four F_2 mother plant.

5. F_3 segregants (200 plants) were grown in a disease sick field and evaluated for their biometric parameters and disease reaction. Out of the 200 plants, 104 plants showed bacterial wilt resistance in the wilt sick field. However, only twelve plants showed combined resistance to bacterial wilt and ToLCV.

6. Analysis of variance indicated superiority in F_2-18 (60.93 cm) with respect to plant height. For days to flowering and fruiting, no significant variation was observed among F_3 segregants.

7. The segregants of F_2-33 showed superiority with respect to yield per plant. The plant number F_2-33-30 gave maximum fruits per plant also. The plant F_2-33-37 gave the highest yield (3281 g/plant) in F_3 population.

8. The six primers selected for polymorphism in the parents (Sakthi and IIHR 2196) were evaluated on the segregating F_3 population (200 plants).

9. Among the six, the SSRKAU 11 and SSR 450 failed to give polymorphism in the segregating lines DNA from all the F_3 lines gave monomorphic amplification pattern.

10. The ISSR primer HB 12 could amplify the specific band for ToLCV resistance in 60 progenies.

11. The primer Ualty 16 reported to be specific for ToLCV resistance was amplified in 52 F_3 segregants lines.

12. Field screening could identify only 12 F_3 lines with ToLCV resistance. Most of the segregating lines with ToLCV resistance would have disappeared from field due to bacterial wilt incidence substantiating the difference between field reaction and marker segregation. Compared to

ISSR marker HB 12, Ualty 16 can be considered as more specific, since it is a SCAR marker.

13. The selected primers LEaat 16 and $TSCAR_{AAG/CAT}$ which were reported to be specific for bacterial wilt identified 130 and 113 segregants respectively as resistant ones. However, in field reaction only 104 F_3 segregants were identified as wilt resistant. This disparity could be explained with other factors like transplanting shock and physical damages at early stages that would have resulted in wilting. Further, such disparity could be expected in F_3 segregating population.

14. The study could identify 12 F_3 lines with combined resistance to bacterial wilt and ToLCV.

15. The F_3 lines of the plant F_2-33 were found superior for yield and disease resistance. Four high yielding lines with combined resistance were detected in this group, while most of the other plants with combined resistance were not good yielders.

16. The present study strongly suggests further evaluation of the selected 12 F_3 lines with combined resistance. The segregation of the four markers identified are also to be validated in further generation (F_4, F_5 etc.).

17. The high yielding lines having combined resistance can be further evaluated for selecting pure lines with high yield and disease resistance.

References

REFERENCES

Abeygunawardena, D. V. W. and Siriwardena, A. A. P. 1963. Studies on resistance in tomato to bacterial wilt. *Trop. Agric.* **119**: 55-66.

Acosta, J. C. 1972. Genetic analysis for bacterial wilt resistance in a tomato cross, *Lycopersicon esculentum* Mill X *L. pimpinellifolium* Mill. *Proc. 3rd Ann. Meeting, Crop. Sci*., **183**-190 pp.

Acosta, J. C., Gilbert, J. C. and Quinon, V. L. 1964. Heritability of bacterial wilt resistance in tomato. *Proc. Am. Soc. Hort. Sci*. **84**: 455-462.

Agrama, H. A. and Scott, J. W. 2006. Quantitative trait loci for tomato yellow leaf curl virus and tomato mottle virus resistance in tomato. *J. Am. Soc. Hort. Sci.* **131**: 267-272.

Alpatev, A. V. 1981. Evolution of the tomato species (*Lycopersicon esculentum* Mill). *Biol*. **16**: 61-64.

Ammiraju, J. S. S., Veremis, J. C., Huang, X., Roberts, P. A. and Kaloshian, I. 2003. The heat-stable root-knot nematode resistance gene *Mi-9* from *Lycopersicon peruvianum* is localized on the short arm of chromosome 6. *Theor. Appl. Genet.* **106(3)**: 478-484.

Anbinder, I., Reuveni, M., Azari, R., Paran, I., Nahon, S., Shlomo, H., Chen, L., Lapidot, M. and Levin, I. 2009. Molecular dissection of tomato leaf curl virus resistance in tomato line TY 172 derived from *Solanum peruvianum*. *Theo. Appl. Genet.*, **119**: 519-530.

Anonymouse. 1998. Bacterial wilt disease: molecular and ecological aspects. (Eds. Prior, P., Allen, C. and Elphinstone, J.). INRA edn, *Springer Verlag*, Berlin (Germany), 447 p.

Areshchenkova, T. and Ganal, M. W. 2002. Comparative analysis of polymorphism and chromosomal location of tomato microsatellite

markers isolated from different sources. *Theor. Appl. Genet.* **104**: 229-235.

AVRDC Report 2000. Asian Vegetable Research and Development Centre, Shanhuna, Taina, Taiwan, *AVRDC Publication no. 01-520*, 152p.

AVRDC Report 2001. Asian Vegetable Research and Development Centre, Shanhuna, Taina, Taiwan, *AVRDC Publication no. 02-542*, 151p.

Bai, Y., Huang, C. C., van der Hulst, R., Meijer-Dekens, F., Bonnema, G. and Lindhout, P. 2003. QTLs for tomato powdery mildew resistance (*Oidium lycopersici*) in *Lycopersicon parviflorum* G1.1601 co-localize with two qualitative powdery mildew resistance genes. *Mol. Plant Microbe. Interact.* **16(2)**: 169-176.

Banejee, M. K. and Kalloo, G. 1989. The inhetance of earliness and fruit weight in crosses between cultivated tomatoes and two wild species of Lycopersicum. Plant Breed. 102: 148-152.

Banerjee, M. K. and Kalloo, G. 1987. Inheritance of tomato leaf curl virus resistance in *Lycopersicon hirsutum*, F. *glabratum. Euphytica*, **36**: 581-584.

Barton, D. W. 1950. Pachytene morphology of the tomato chromosome complement. *Am. J. Bot.* **37**: 639-643.

Barton, D. W., Butler, L., Jenkins, J. A., Rick, C. M. and Young, P. A. 1955. Rules for nomenclature in tomato genetics. *J. Hered*. **46**: 22-26.

Ber, R., Navot, N., Zamir, D., Antignus, Y., Cohen, S. and Czosnek, H. 1990. Infection of tomato yellow leaf curl virus: susceptibility to infection, symptom development, and accumulation of viral DNA. *Arch. Virol.* **112**: 169-180.

Bertrand C., Collard Y. and Mackill, D. J. 2007. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Biol. Sci.* **363**: 557-572.

Bhushana, H. O. 2000. Molecular detection, genetics of tomato leaf curl virus (ToLCV) resistance, fruit yield, its attributes and fruit quality trait in tomato. Ph. D. dissertation thesis, University of Agricultural Sciences, Bangalore, 150 p.

Bosch, S. E., Louw, A. J. and Aucamp, E. 1985. "Rodade", bacterial wilt resistant tomato. *Hort. Science* **20**: 458-459.

Boswell, V. R. 1949. Our vegetable travellers. The National Geographic magazine. August, Xc **VI**: 145-217.

Brouwer, D. J. and St. Clair, D. A. 2004. Fine mapping of three quantitative trait loci for late blight resistance in tomato using near isogenic lines (NILs) and sub-NILs. *Theor. Appl. Genet.* **108**: 628–638.

Buddenhagen, I. W. and Kelman, A. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. *Ann. Rev. Phytopath*. **2**: 203-226.

Buddenhagen, I. W., Sequeria, L. and Kelman, A. 1962. Designation of races *Pseudomonas solanacearum*. *Phytopathol.* **52**: 726.

Butler, L. 1951. New linkage map of the tomato. *J. Hered*. **42**: 100-104.

Cao, B. H., Lei, J. J, Wang, Y and Chen, G. J. 2009. Inheritance and identification of SCAR marker linked to bacterial wilt-resistance in eggplant. *African J. Biotechnol*. **8(20)**: 5201-5207.

Carmeille, A., Caranta, C., Dintinger, J., Prior, P., Luisetti, J. and Besse, P. 2006. Identification of QTLs for *Ralstonia solanacearum* race 3-phylotype II resistance in tomato. *Theor. Appl. Genet.* **113**: 110-121.

Celine, V. A. 1981. Genetic cataloguging of tomato germplasm towards isolation of line(s) resistant to bacterial wilt. M. Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, p. 69.

Chague, V., Mercier, J. C., Guenard, M., deCourcel, A. and Vedel, F. 1997. Identification of RAPD markers linked to a locus involved in quantitative resistance to TYLCV in tomato by bulked segregant analysis. *Theor. Appl. Genet.* **95**: 671–677.

Channarayappa, A. and Shivashankar, G. 1992. Resistance of *Lycopersicon* species to Bemisia tabaci, a tomato leaf curl virus vector. *Can. J. Bot*. **70**: 2184–2192.

Chester, K. S. 1950. *Nature and Prevention of Plant Diseases* (2nd edn.), McGraw Hill Book Co. Inc., New York, p. 525.

Chupp, C. and Sherf, A. F. 1960. Vegetable Disease and Their Control, The Ronald Press Co., New York, 695p.

Cohen, S. and Harpaz, I. 1964. Periodic rather than continual acquisition of new tomato virus by its vector, the tobacco whitfly (*Bemesia tabaci* Gennadus). *Ent. Exp. Appl.* **7**: 155-166.

Collard, B. C. Y., Jahufer, M. Z. Z., Brouwer, J. B. and Pang, E. C. K. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and markerassisted selection for crop improvement: The basic concepts. *Euphytica* **142**: 169-196.

Czosnek, H. and Laterrot, H. 1997. A worldwide survey of tomato yellow leaf curl viruses. *Arch. Virol*. **142**: 1391-1406.

Danesh, D., Aarons, S., McGill, G. E. and Young, N. D. 1994. Genetic dissection of oligogenic resistance to bacterial wilt in tomato. *Mol. Plant-Microb. Interact.* **7**: 464-471.

Dhaliwal, M. S., Surjan, S. and Cheema, D. S. 2003. Line x Tester analysis for yield and processing attributes in tomato. *J. Punjab. Res. Punjab Agrc. Uni.* **40 (1)**: 49-53.

Dickinson, M. J., Jones, D. A. and Jones, J. D. G. 1993. Close linkage between the *Cf*-2/*Cf*-5 and *Mi* resistance loci in tomato. *Mol Plant Microb. Inter.* **6**: 341-347.

Dixit, J., Kalloo, G., Bhutani, R. D. and Sidhu, A. S. 1980. Line x tester analysis for the study of heterosis and combining ability in tomato. *Haryana J. Hort. Sci*. **9**: 56-61.

Dry, I. B., Rigden, J. E., Krake, L. R., Mullineaux, P. M. and Rezaian, M. A. 1993. Nucleotide sequence and genome organization of tomato leaf curl geminivirus. *J. Gen. Virol.* **74**: 147-151.

Eastwood, R. F., Lagudah, E. S. and Appels, R. 1994. A directed search for DNA sequences tightly linked to cereal cyst nematode resistance genes in *Triticum tauschii*. *Genome* **37**: 311-319.

Edwards, J. D. and McCouch, S. R. 2007. Molecular markers for use in plant molecular breeding and germplasm evaluation. In: Guimaraes, E. P., Ruane, J., Scherf, B. D., Sonnino, A. and Dargie, J. D. (Eds.) Marker-assisted selection. Current status and future perspectives in crops, livestock, forestry and fish. Food and Agriculture Organization of the United Nations, Rome, 29-49 p.

Estelitta, S., Nair, P. V., Vilasini, T. N. and Sankar, J. 1997. *Moringa oleifera* new host of *Ralstonia solanacearum* (E. Smith) from India. *ACIAR Bacterial Wilt Newsettl*., **14**: 6.

Fegan, M. and Prior, P. 2005. How coplex is the "Ralstonia solanacearum species complex"? In: Allen C, Prior P and Hayward, A. C. (eds) Bacterial wilt:

The disease and *Ralstonia solanacearum* species complex. APS Press, St. Paul, 449-461p.

Filho, M. H. and Stevens, M. 1980. Tomato breeding for nematode resistance: survey of resistant varieties for horticultural characteristics and genotype of acid phosphatase. *Acta Hort.* **100**: 383-391.

Flores, E. M. and Espinoz, A. M. 1977. Leaf morphology of Lycopersicon esculentum. *Revista de Biologia Tropical* **25**: 289-299.

Foolad, M. R. and Panthee, D. R. 2012. Marker-Assisted Selection in Tomato Breeding. *Critical Reviews in Plant Sciences* **31**: 93-123.

Fortnum, B. A. and Martin, S. B., 1998, Disease management strategies for control of bacterial wilt of tobacco in the south-eastern USA. In : *Bacterial Wilt Disease : Molecular and Ecological Aspects,* Prior, P., Allen, C. and Elphinstone, J. G. Eds. Springer-Verlag, Berlin, Germany, pp. 394-402.

Frary, A., Xu, Y., Liu, J., Mitchell, S., Tedeschi, E. and Tanksley, S. D. 2005. Development of a set of PCR-based anchor markers encompassing the tomato genome and evaluation of their usefulness for genetics and breeding experiments. *Theor. Appl. Genet.* **111(2)**: 291-312.

Gameel, O. L. 1977. A new discussion, distribution and host of cotton whitfly *Bemisia tabaci* (Homoptera: Aleyroidae). *Revude de Zoologie. et de Botanieque Africanies*, **86**: 56-64.

Geethanjali, S., Chen, K. Y., Pastrana, D. V. and Wang, J. F. 2010. Development and characterization of tomato SSR markers from genomic sequences of anchored BAC clones on chromosome 6. *Euphytica*, **173**: 85-97.

Gilbert, G. S. 2002. Evolutionary ecology of plant diseases in natural ecosystems. *Phytopathol.* **40**: 13-43.

Gilbert, J. C., Tanaka, J. and Takeda, K. Y. 1973. 'Kewalo' tomato. Hort. Science **9**: 481-482.

Gonza´lez-Cabezuelo, J. M., Capel, J., Abad, J., Toma´s, D. M., Ferna´ndez-Mun˜oz, R., Moriones, E. and Lozano, R. 2012. Genotyping selection for resistance against tomato yellow leaf curl virus (TYLCV) conferred by *Ty-1* and *Ty-3* genes in tomato. *Mol. Breed.* **30**: 1131-1142.

Goodman, W. G. 1977. Single stranded DNA genome in a whitfly transmitted plant virus. *Virology* **43**: 171-179.

Goulao, L. and Oliveira, C. M. 2001. Molecular characterization of cultivars of apple (*Malus domestica*) using microsatellite (SSR and ISSR) markers. *Euphytica* **122**: 81-89.

Grimault, V., Prior, P. and Anais, G. 1995. A monogenic dominant resistance of tomato to bacterial wilt in Hawaii-7996 is associated with plant colonization by *Pseudomonas solanacearum*. *J. Phytopathol.* **143**: 349-352.

Gupta, M., Chyi, Y. S., Romero-Sevenson, J. and Owen, J. L. 1994. Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple sequence repeats. *Theor. Appl. Genet.* **8**: 998-1003.

Hanson, P. M., Bernacchi, D. and Green, S. 2000. Mapping a wild tomato introgression associated with tomato yellow leaf curl virus resistance in a cultivated tomato line. *J. Am. Soc. Hortic. Sci.* **125(1)**: 15-20.

Hanson, P. M., Licardo, O., Hanudin Wang, J. F. and Chen, J. T. 1998. Diallel analysis of bacterial wilt resistance in tomato derived from different sources. *Plant Dis.* **82**: 74-78.

Hartl, L., Weiss, H., Stephan, U., Zeller, F. J. and Jahoor, A. 1995. Molecular identification of powderymildew resistance genes in common wheat (*Triticum aestivum* L.). *Theor. Appl. Genet.* **90**: 601-606.

Hassan, A. A., Laterrot, H., Mazyed, H. M., Moustafa, S. E. and Desouki, I. A. M. 1985. Yield response of some tomato cultivars to artificial inoculation with tomato yellow leaf curl virus. *Egyptian J. Hort.* **12**: 55-60.

Hayati, J. 1978. Some investigation on control of tomato leaf curl virus in Haryana. M. Sc. (Ag.) thesis, Haryana Agricultural University, Hissar, 113p.

Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum*. *J. Appl. Bact*. **27**: 265-277.

Hayward, A. C. 1994. Systematics and phylogeny of *Pseudomonas solanacearum* and related bacteria. Bacterial wilt- The disease and it's Causative Agent *Pseudomonas solanacearum* (eds. Hayward, A. C. and Hartman, G. L.), CAB International, UKP, pp. 123-133.

He, C., Poysa, V. and Yu, K. 2003. Development and characterization of simple sequence repeat (SSR) markers and their use in determining relationships among *Lycopersicon esculentum* cultivars. *Theor. Appl. Genet*. **106**: 363-373.

Hedayathulla, S. and Saha, J. C. 1941. Bacterial wilt disease of tomato. *Sci. Cult.* **7**: 226-227.

Henderson, U. P. and Jenkins, S. F. 1972. Venus and Satum: two new tomato varieties combining desirable horticultural m features with Southern bacterial wilt resistance. *Bull. Agric. Exp. Stn. North Carolina State Univ*. **444**: 13.

Hernandez, M. J. and Oyaszum, J. C. 2006. Two minipreperation protocol to DNA extraction from plant with high polysaccharide and secondary metabolites. *Afr. J. Biotechnol.* **5(20)**: 1864-1867.

Hildebrant, A. C. 1950. Some important galls and wilt of plants and the inciting bacteria. *Biol. Rev*. **14**: 259-272.

Humphrey, L. M. 1934. The meiotic division of haploid, diploid and tetraploids tomatoes with special reference to the prophase. *Cytologia* **5**: 278-300.

Huskins, S. 1934. Anomalous segregation of a triploid tomato. *J. Hered*. **25**: 281-286.

Hussain, A. and Kelman, A. 1957. Presence of piece and cellulolytic enzymes in tomato plants infected by *Pseudomonas solanacearum*. *Phytopathol* **47**: 111-112.

Jacob, H. J., Lindpaintner, K., Lincoln, S. E., Kusumi, K., Bunker, R. K., Mao, Y. P., Ganten, D., Dzau, V. J. and Lander, E. S. 1991. Genetic mapping of a gene causing hypertension in the stroke-prone spontaneously hypertensive rat. *Cell* **67**: 213-224.

Jenkins, J. A. 1948. The origin of the cultivated tomato. *Econ. Bot*. **2**: 379-392.

Ji, Y. F., Scott, J. W. and Schuster, D. J. 2009a. Toward fine mapping of the tomato yellow leaf curl virus resistance gene *Ty-2* on chromosome 11 of tomato. *Hort. Science* **44**: 614-618.

Ji, Y. F., Scott, J. W., and Schuster, D. J. 2009b. Molecular mapping of *Ty-4*, a new tomato yellow leaf curl virus resistance locus on chromosome 3 of tomato. *J. Amer. Soc. Hort. Sci.* **134**: 281-288.

Jones, D. A., Dickinson, M. J., Balint-Kurti, P. J., Dixon, M. S. and Jones, J. D. G. 1993. Two complex resistance loci revealed in tomato by classical and

RFLP mapping of *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* genes for resistance to *Cladosporium fulvum*. *Mol. Plant Microb. Interact.* **6**: 348-357.

Jones, D. F. 1917. Linkage in *Lycopersicon*. *Am. Nat*. **51**: 608-621.

Jyothi, H. K., Santhoshal, H. M. and Basamma. 2012. Recent advances in breeding for bacterial wilt (*Ralstonia solanacearum*) resistance in tomato-review. *Curr. Biotica*. **6 (3)**: 370-398.

Kallo, G. and Banrjee, M. K. 2000. H-24 moderately leaf curl resistant variety of tomato (*Solanum lycopersicum* L.). *Veg. Sci*. **27**: 117-120.

Kao, J. Y. and Hsiao, J. V. 1980. A study on the relationship between the phenolic contents of tomato cultivars and the resistance or susceptibility of tomato cultivars to bacterial wilt disease. *J. Sci. Engg*. **17**: 295-305.

Karumannil, S., Sadhankumar, P. G., Nazeem, P. A., Girija, D. and Kesavachandran, R. 2008. DNA finger printing of bacterial wilt resistant tomato (*Solanum lycopersicum* L.) cultivars. *Veg. Sci*. **35(1)**: 105-108.

KAU [Kerala Agricultural University] 2011. Package of Practices Recommendations: Crops. Kerala Agricultural University Press, Thrissur, 334p.

Kelman, A. 1953. The relationship of pathogenicity of *Pseudomonas solanacearum.* A literature review and bibliography. *North Carolina Agric. Exp. Stn. Tech. Bull*. **99**: 194.

Khush, G. S. and Rick, C. M. 1967. Studies on the linkage map of chromosome 4 of the tomato and on the transmission of induced deficiencies. *Genetica* **38**: 74-94.

Klein-Lankhorst, R., Rietveld, P., Machiels, B., Verkerk, R., Weide, R., Gebhard, T. C., Kornneef, M. and Zabel, P. 1991. RFLP markers linked to the root

knot nematode resistance gene *Mi* in tomato. *Theor. Appl. Genet.* **81**: 661-667.

Yadav, K. 2011. Incorporation of tomato leaf curl virus (ToLCV) resistance in bacterial wilt resistant tomato. M. Sc. (Hort.) thesis, Kerala Agriculture University, Thrissur.

Lazarowitz, S. G. 1992. Gemimiviruses: genome structure and gene function. *Crit. Rev. Plant Sci*. **11**: 327-349.

Lesley, J. W. 1928. A cytological and genetical study of progenies of triploid tomatoes. *Genetics* **17**: 545-559.

Li, Y., Dong Y. B., Niu, S. Z. and Cui, D. Q. 2008. QTLs for popping characteristics in popcorn. *Plant Breed.* **126**: 509-514.

Lin, D., Hubbes, M. and Zsuffa, L. 1993a. Differentiation of poplar and willow clones using RAPD fingerprints. *Tree Physiol.* **14**:1097-1105.

Lindstrom, E. W. 1941. A haploid mutant in the tomato. *J. Hered*. **20**: 23-30.

Lindstrom, E. W. and Humphrey, L. M. 1932. Comparative cytogenetic studies of tetraploids tomatoes from different origins. *Proc. 6th Int. Cong. Genet. N. Y*. **2**: 118-119.

Lucas, G. B. 1975. Granville wilt. In: *Disease of Tobacco 3rd* edi. Biological Consulting Association, Raleigh NC., pp. 365-382.

MacArthur, J. W. 1923. Linkage studies with the tomato. *Genetics* **11**: 387-405.

Mangin, B., Thoquet, P., Olivier, J. and Grimsley, N. H. 1999. Temporal and multiple quantitative trait loci analyses of resistance to bacterial wilt in tomato permit the resolution of linked loci. *Genetics* **151**: 1165-1172.

Mansour, A. and Al-Musa, A. 1992. Tomato yellow leaf curl virus: host range and virus-vector relationship. *Plant Pathol*. **41**: 122-125.

Mathew, S. K. 2001. Biocontrol of Ralstonia solanacearum E. F. Smith causing bacterial wilt in solanaceous vegetable crops, (ICAR Project) Annual Report, Kerala Agricultural University, Thrissur, p.28.

Mayee, C. D., Kanwar, J. S. and Nandapuri, K. S. 1974. The comparative performance of different genotypes of tomato vis-vis leaf curl and mosaic. *J. Res. Panjab Agric. University* **11**: 362-364.

McClean, P. L, Ewing, J. Lince, M. and Grafton, K.1994. Developments of RAPD map of *Phaseoolus vulgaris* L. *Annual Report of the bean improvement Cooperative* **37**: 79-80.

McCue, G. A. 1952. The history of the use of tomato. An annotated bibliography. *Ann. Rept. Mp. Bot. Gard*. **39**: 299-348.

MeKsem, K., Leister, D., Peleman, J., Zabeau, M., Salamini, F., and Gebhardt, C. 1995. A high-resolution map of the vicinity of the *R1* locus on chromosome V of potato based on RFLP and AFLP markers. *Mo.l Gen. Genet.* **249**: 74-81.

Melton, T. A., Broadwell, A. and Wilson, J. 2004. Disease management. In: *Flue-Cured Tobacco Information,* North Carolina Cooperation Extension Service. NC, pp. 142-166.

Mew, T. W. and Ho, W. C. 1977. Effect of soil temperature on resistance of tomato cultivars to bacterial wilt. *Phytopathol.* **67**: 909-911.

Mew, T. W. and Ho,W. C. 1976. Varietal resistance to bacterial wilt in tomato. *Plant Disease Report*. **60**: 264-268.

Miao, L. X., Shou, S. Y., Cai, J. Y., Jiang, F., Zhu, Z. J. and Li, H. B. 2009. Identification of two AFLP markers linked to bacterial wilt resistance in tomato and conversion to SCAR markers. *Mol. Biol. Rep.* **36**: 479-486.

Mohan, M., Nair, S., Bentur, J. S., Prasada-Rao, U. and Bennett, J. 1994. RFLP and RAPD mapping of the rice *Gm2* gene that confers resistance to biotype 1 of gall midge (*Orseolia oryzae*). *Theor. Appl. Genet.* **87**: 782-788.

Moreau, P., Thoquet, P., Olivier, J., Laterrot, H., and Grimsley, N. 1998. Genetic mapping of *Ph-2*, a single locus controlling partial resistance to *Phytophthora infestans* in tomato. *Mol. Plant-Microbe Interact.* **11**: 259-269.

Morgante, M. and Olivieri, A. M. 1993. PCR-amplified microsatellites as markers in plant genetics. *Plant J.* **3**: 175-182.

Moustafa, S. E. S. and Hassan, A. A. 1993. Tomato cv. Evolution with emphasis on tomato yellow leaf curl virus tolerance. *Assuit. J. Agric. Sci*. **24**: 255-272.

Mueller, L. A., Lankhorst, R. K., Tanksley, S. D. and Giovannoni, J. J. 2009. A snapshot of the emerging tomato genome sequence. *Plant Genome* **2**: 78-92.

Mueller, L. A., Solow, T. H., Taylor, N., Skwarecki, B., Buels, R., Binns, J., Lin, C., Wright, M. H., Ahrens, R., Wang, Y., Herbst, E. V., Keyder, E. R., Menda, M., Zamir, D. and Tanksley, S. T. 2005. The SOL Genomics Network; A Comparative Resource for Solanaceae Biology and Beyond. *Plant Physiolog*. **138**: 1310-1317.

Muniyappa, V., Jalikop, S. H., Saikia, A. K., Chennarayappa, Shivashankar, G., Ishwara, A. and Ramappa, H. K. 1991. Reaction of *Lycopersicon* cultivars and wild accessions to tomato leaf curl virus. *Euphytica* **56**: 37–41.

Naidu, D. Y. 1993. Study of segregating population in tomato (*Solanum lycopersicu*m L.). M. Sc. (Ag.) thesis, University of Agricultural Sciences, Dharwad, 140p.

Nair, S., Bentur, J. S., Prasada-Rao, U. and Mohan, M. 1995. DNA markers tightly linked to a gall midge resistance gene (*Gm2*) are potentially useful for marker-aided selection in rice breeding. *Theor. Appl. Genet.* **91**: 68-73.

Nair, S., Kumar, A., Srivastava, M. N. and Mohan, M. 1996. PCR-based DNA markers linked to a gall midge resistance gene, *Gm4t*, has potential for marker aided selection in rice. *Theor. Appl. Genet.* **92**: 660-665.

Nakhla, M. K., Maxwell, D. P., Martinez, R. T., Carvalho, M. G. and Gilbertson, R. L. 1994. Occurrence of the Eastern Mediterranean strain of tomato yellow leaf curl geminivirus in the Dominican Republic. *Phytopathol.* **84**: 1072.

Naqvi, N. I., Bonman, J. M., Mackill, D. J., Nelson, R. J. and Chattoo, B. B. 1995. Identification of RAPD markers linked to a major blast resistance gene in rice. *Mol. Breed.* **1**: 341-348.

Nayar, K. 1982. Etiology, survival and control of bacterial wilt of brinjal caused by *Pseudomonas solanacearum* E. F. Smith., M. Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 150p.

Nazeem, P. A., Girija, D., Sadhankumar. P. G., Nirmaladevi, S., Mathew. S. K., and Umamaheshwaran, 2010. Isolation and characterization of gene encoding disease resistance (ToLCV and bacterial wilt) in tomato. Final report of Sol Genome Project (KAU), submitted to DBT, Govt. of India.

Nelson, R. R. 1974. The meaning of disease resistance in plant. In Nelson R. R. (Ed.), Breeding Plant for Disease Resistance-Concept and Application. The Penn State Univ. Press, pp. 13-25.

NHB [National Horticulture Database]. 2014. NHB home page [on line]. Available: http://www.nhb.gov.in . [2 July 2014].

Nienhuis, J., Helentjaris, T., Slocum, M., Ruggero, B., and Schaefer, A. 1987. Restriction fragment length polymorphism analysis of loci associated with insect resistance in tomato. *Crop Sci*. **27**: 797-803.

Nirmala Devi, S. 1987. Studies on genetic resistance to bacterial wilt (*Pseudomonas solanacearum* E. F. Smith) and root-knot nematode (*Meloidogyne incognita*) in tomato (*S. lycopersicum* L.) Ph. D. thesis, University of Agricultural Sciences, Bangalore, 122p.

Ohyama, A., Asamizu, E., Negoro, S., Miyatake, K., Yamaguchi, H., Tabata, S. and Fukuoka, H. 2009. Characterization of tomato SSR markers developed using BAC-end and cDNA sequences from genome database. *Mol. Breed.* **23**: 685-691.

Opena, R. T. 1985. Development of tomato and Chinese cabbage cultivars adapted to the hot, humid tropics. *Acta. Hort.* **153**: 421-436.

Opena, R. T., Kuo, G. C. and Yoon, J. Y. 1987. Breeding for stress tolerance under tropical conditions in tomato and heading Chinese cabbage. In: *Improved Vegetable Production in Asia.* ASPAC. FFTC. Taipei, Taiwan, 36. pp. 88-109.

Osborn, B., Lightfoot, D. R. and Hill, A. 2005. Assessment of genetic polymorphism of *Pinus ponderosa* by Inter Simple Sequence Repeats (ISSR). *J. Sci*. **1**: 46-51.

Osborn, T. C., Alexander, D. C. and Fobes, J. F. 1987. Identification of restriction fragment length polymorphisms linked to genes controlling soluble solids content in tomato fruit. *Theor. Appl. Genet.* **73**: 350-356.

Panthee, D. R. and Foolad, M. R. 2012. A re-examination of molecular markers for use in marker-assisted breeding in tomato. *Euphytica* **184**: 165-179.

Patil, V. R. J. 2001. Studies on genetics of bacterial wilt resistance and heterosis for yield and quality attributes in tomato (*Solanum lycopersicum* L.). M. Sc. (Ag.) thesis, University of Agricultural Sciences, Bangalore, 110p.

Polston, J. E., Bois, D., Serra, C. A. and Concepci!on, S. 1994. First report of a tomato yellow leaf curl-like geminivirus from tomato in the Western hemisphere. *Plant Dis*. **78**: 831.

PPV and FRA [Protection of Plant Varieties and Farmers Rights Authority]. Guidelines for the conduct of test for distinctiveness, uniformity and stability. PPV and FRA, New Delhi, 12p.

Prabhushankar, H. R. 1990. Genetic analysis of yield components in tomato (*Solanum lycopersicum* L.), M. Sc. (Ag.) thesis, University of Agricultural Sciences, Dharwad, 140p.

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

Ramachandran, C., Gopalakrishan, T. R. and Peter, K. V. 1980. Evolving high yielding tomato varieties with resistance to bacterial wilt. *Annual Report*, Kerala Aricultural University, Thrissur. 181p.

Rick, C. M. 1969. Origin of the cultivated tomato. Current status of the problem. *Internat. Bot.* **XI**: 180.

Rick, C. M. 1971. Some cytogenetic features of the genome in diploid plants species. *Stadler Symposia* **1** and **2**: 153-174.

Rick, C. M. 1976. Tomato (Family Solanaceae) In: Simmond, N. W. (Ed.) Evolution of Crop Plants, Longman, London.

Rick, C. M. 1979. The tomato. *Sci. Am*. **239**: 67-76.

Rick, C. M. 1982. Linkage map of the tomato *Lycopersicon esculentum*. *Genetic. Maps* **2**: 360-367.

Rick, C. M. and Fobes, J. H. 1975. Allozyme Variation in the cultivated tomato and closely related species. *Bull. Torr. Bot. Club*. **102**: 376-384.

Rick, C. M. and Khush, G. S. 1969. Cytogenetic exploration in the tomato genome. *Genet. Lett.* **1**: 45-68.

Rick, C. M. and Zobel, R. W. 1972. New linkage testers. *Rep. Tomato Genet. Coop*. **22**: 24.

Rogers, S. O. and Bendich, A. J. 1994. Extraction of total cellular DNA from plants, algae and fungi; In: Gelvin, S. B. and Schilperoort, R. A. (eds.), *Plant Molecular Biology manual*, MA Kluwer Academic Publishers, Boston, pp. 1-8.

Ronald, P. C., Albano, B., Tabien, R., Abenes, L., Wu, K. S., McCouch, S. and Tanksley, S. D. 1992. Genetic and physical analysis of the rice bacterial blight disease resistance locus, *Xa21*. *Mol Gen Genet* **236:** : 113-120.

Sadashiva, A. T., Reddy, K. M., Reddy, M. K., Singh, T. H., Balaram, M. V., Narasimha Prasad, B. C., Prasanna, K. M., Naveen, L. R. and Joshi, S. C. 2003. Tomato leaf curl virus and bacterial wilt tomato (*Solanum lycopersicum* L.). *Indian J. Plant Genet. Resour*. **16(3)**: 257-258.

Sadashiva, A. T., Reddy, M. K., Ravishankar, K. V., Reddy, K. M., Singh, T. H., Naveen, L. R., Gunavantha, J., Ravi, B. A. and Joshi, S. G. 2007. Identification of stable sources of resistance to tomato leaf curl virus in tomato (*Solanum lycopersicum*). Plant and Animal Genome XV Conference, January 13-17, 2007, Town and Country Convention Centre, San Diego, CA, p.928.

Sadhankumar, P. G. 1995. Incorporation of resistance to fruit cracking in a bacterial wilt resistant background in tomato. Ph. D thesis, Kerala Agricultural University, Vellanikkara, Thrissur, 146p.

Saiga, A. K. and Muniyappa, V. 1989. Epidemilogy and control of tomato leaf curl virus in Southern India. *Tropica Agriculture (Trinidad)* **66**: 350-354.

Saiki, R. K., Gelfand, D. H., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermo stable DNA polymerase. *Science* **239**: 487-491.

Sambasivam, P. K. 2003. Characterisation of *Ralstonia solanacearum* Yabuuchi *et al*., causing bacterial wilt in ginger using molecular marker. M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, p.67.

Sandbrink, J. M., van Ooijen, J. W., Purimahua, C. C., Vrielink, M., Verkerk, R., Zabel, P. and Lindhout, P. 1995.Localization of genes for bacterial canker resistance in *Lycopersicon peruvianum* using RFLPs. *Theor. Appl. Genet.* **90**: 444-450.

Sarfatti, M., Abu-Abied, M., Katan, J. and Zamir, D. 1991. RFLP mapping of *I1*, a new locus in tomato conferring resistance against *Fusarium oxysporum* f. sp. *lycopersici* race 1. *Theor Appl Genet* **82**: 22-26.

Sastry, K. S. and Singh, S. J. 1973. Assessment of loses in tomato by tomato leaf curl virus. *Indian J. Hort*. **35**: 50-54.

Sastry, K. S. M., and Singh, S. J. and Sastry, K. S. 1978. Studies on epidemiology of tomato leaf curl virus. *Indian J. Mycol. Plant Pathol*. **3**: 50-54.

Schachermayr, G. M., Messmer, M. M., Feuillet, C., Winzeler, H., Winzeler, M. and Keller, B. 1995. Identification of molecular markers linked to the *Agropyron elongatum*-derived leaf rust gene *Lr24* in wheat. T*heor Appl Genet.* **90**: 982-990.

Schachermayr, G., Siedler, H., Gale, M. D., Winzeler, H., Winzeler, M. and Keller, B. 1994. Identification and localization of molecular markers linked to the Lr9 leaf rust resistance of wheat. *Theor. Appl. Genet.* **88**: 110-115.

Schaub, I. O. and Baver, L. D. 1944. Research and farming. *67th Reptr. North Carolina Agric. Exp. Stn.* **4**: 27-31.

Scott, J. W. 2007. Breeding for resistance to viral pathogens. In: Genetic Improvement of Solanaceous Crops Vol. 2: Tomato. M. K. Razdan and Antur K. Mattoo Eds. Science Publishers, Enfield, USA. 457-485.

Scott, J. W., Somodi, G. C. and Jones, J. B. 1988. Bacterial spot resistance is not associated with bacterial wilt resistance in tomato. *Proc. Fla. State Hort. Soc.* **101**: 390-392. less

Scott, J. W., Wang, J. F. and Hanson, P. M. 2005. Breeding tomatoes for resistance to bacterial wilt, a global view. *Proc. 1st Intl. Symp. Tomato Dis.* **695**: 161-172.

Sequeira, L. 1993. Bacterial wilt: Past, present and future. *ACIAR Proceedings* **45**: 12-21.

SGN [Sol Genomics Network]. 2014. SSGN home page [on line]. Available: http://www.sng.cornell.edu. [10 Oct 2012].

Shekhawat, G. S., Kishore, V., Singh, D. S., Khanna, R. N., Sighar and Bahal, V. K. 1979. Survival at *Pseudomonas solanacearum* under diverse agroclimate in India. *Indian Journal of Agricultural Sciences* **49**: 736-738.

Shew, H. D. and Lucas, G. B. 1991. *Compendium of Tobacco Diseases*, APS Press. MN, USA. p68.

Shoba, N. and Armugaum, R. 1991. Association of leaf curl virus resistance in tomato. *South Indian Hort*. **39**: 281-285.

Singh, K. 1961. Inheritance of North Carolina type of bacterial wilt resistance in tomato. *Lycopersicon esculentum* L. M. Sc. thesis, University of Hawaii.

Singh, T. H. and Sadashiva, A. T. 2007. Identification of heat tolerant (HT) and tomato leaf curl virus (ToLCV) resistant lines in tomato (*Solanum lycopersicum* L.) *Abstract; International Conference of Indian Virological Society on "Emerging and Re-emerging viral Diseases of the Tropics and Subtropics",* December 11-14, 2007, Indian Agricultural Research Institute, New Delhi, India.

Siri, M. I., Galván, G. A., Quirici, L., Silvera, E., Villanueva, P., Ferreira, F., Franco F. L. and Pianzzola, M. J. 2009. Molecular marker diversity and bacterial wilt resistance in wild *Solanum commersonii* accessions from Uruguay. *Euphytica* **165**: 371-382.

Smith, P. G. 1996. A bacterial disease of the tomato, eggplant and Irish potato (*Bacillus solanacearum* Nov. Sp.). US *Dept. Agric. Div. Veg. Phys. Pathol. Bull*. **12**: 1-28.

Smulders, M. J., Bredemeijer, G., Rus-Kortekaas, W., Arens, P. and Vosman, B. 1997. Use of short microsatellites from database sequences to generate polymorphisms among Lycopersicon esculentum cultivars and accessions of other *Lycopersicon* species. *Theor. Appl. Genet.* **97**: 264-272.

Sonoda, R. M. Augustine, J. J. and Volin, R. B. 1979. Bacterial wilt of tomato in Florida: history status, and sources of resistance. *Proc. Fla. State Hort. Sci.* **92**: 100-102.

Tahat, M. M. and Sijam, K. 2010. *Ralstonia solanacearum*: The bacterial wilt causal agent. *Asian. J. Plant Sci*. **9(7)**: 385-393.

Tanksley, S. D. and Hewitt, J. 1988. Use of molecular markers in breeding for soluble solids content in tomato a re-examination. *Theor. Appl. Genet.* **75**: 811-823.

Tanksley, S. D., Ganal, M. W., Prince, J. P., de Vicente, M. C., Bonierbale, M. W., Broun, P, Fulton, T. M., Giovannoni, J. J., Grandillo, S., Martin, G. B., Messeguer, G. B., Miller, J. C., Miller, L., Paterson, A. H., Pineda, O., Riider, M. S., Wing, R. A., Wu, W. and Young, N. D. 1992. High density molecular linkage maps of the tomato and potato genomes. *Genet. Society America*. **132**: 1141-1160.

Taylor, G. R. 1991. Polymerase chain reaction and automation. In: McPerson, M. J., Quirke, P. and Taylor, G. R. (Eds.), *PCR Practical Approach*, Oxford University. Oxford, England. pp 1-14.

Terzopoulos, P. J. and Bebeli, P. J. 2008. DNA and morphological diversity of selected Greek tomato (*Solanum lycopersicum* L.). *Sci. Hortic.* **116(4)**: 354-361.

Thoquet, P., Olivier, J., Sperisen, C., Rogowsky, P., Laterrot, H., and Grimsley, N. 1996. Quantitative trait loci determining resistance to bacterial wilt in tomato cultivar Hawaii 7996. *Mol. Plant-Microbe Interact.* **9**: 826-836.

Tikoo, S. K. 1987. Breeding tomatoes for processing in India. Present status and future prospects. *Acta. Hort.* **200**: 73-81.

Tikoo, S. K., Anand, N., Kishun, R. and Reddy, P. P. 1990. Breeding for combined resistance to bacterial wilt and rooknotnematode in tomato. In *Tomato and Pepper Production in the Tropics.* Green, S. K., Griggs, T. and McLean, B. T. (Eds.), AsianVegetable Research and Development Center. Tainan, Taiwan. p. 99-106.

Van Heusden, A. W., Koornneef, M., Voorrips, R. E., Bruggemann, W., Pet, P., VanVrielink-Ginkel, R. V., Chen, X. and Lindhout, P. 1999. Three QTLs

from *Lycopersicon peruvianum* confer a high level of resistance to *Clavibacter michiganensis* ssp. *michiganensis*. *Theor. Appl. Genet.* **99**: 1068-1074.

Vasudeva, R. S. and Samraj, J. 1948. A leaf curl disease of tomato. *Phytopathol.* **38**: 364-369.

Villareal, R. L. and Lai, S. M. 1979. Development of heat-tolerant tomato varieties in the tropics. In *Proc. 1st International Symposium on Tropical Tomato.* Cowell, R. (Eds.). Aisan Vegetable Research and Development Center. Shanhua, Taiwan. p. 188-200.

Vudhivanich, S. 1997. Infecting filtrations of *Pseudomonas solanacearum* on tomato. *ACIAR Bacterial Wilt Newslett*. **16**: 22.

Walker, J. C. 1952. Disease of Vegetable Crops (1st edn.). McGraw Hill Book Co., New York, p. 529.

Wang, J. F. and Berke, T. 1997. Sources of resistance to bacterial wilt in *Capsicum annum*. *ACIAR Bacterial Wilt Newslett.* **14**: 3-4.

Wang, J. F., Olivier, J., Thoquet, P., Mangin, B., Sauviac, L. and Grimsley, N. H. 2000. Resistance of tomato line Hawaii 7996 to *Ralstonia solanacearum* in Taiwan is controlled mainly by a major strain-specific locus. *Mol. Plant-Microbe Interact.* **13**: 6-13.

Ward, G. M. 1964. Observation of root growth in the tomato plant. *Can. J. Plant. Sci*. **44**: 492-494.

Winkler, H. 1909. Under die *Machkommenschaft* der Solanum *Pfropfbastarde* and die chromosome *nzahlenihyer keimzelien*. *Z. Botan*. **2**: 1-38.

Winkler, H. 1916. Uber die experimentelle Erzeugung Von Pflanzen mit abweichenden chromosomenzahlen. *Z. Boyan*. **8**: 417-531.

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H. and Nishiuchi, Y. 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov. proposal of *Ralstonia solanacearum* (Smith, 1896) Comb. nov. and *Ralstonia eutropha* (Davis, 1969) *Comb. Nov. Microbiol. Immunol*. **39**: 897-904.

Yang, W. and Francis, D. M. 2007. Genetics and breeding for resistance to bacterial diseases in tomato: Prospects for marker-assisted selection. In: *Genetic Improvement of Solanaceous Crops. Vol 2. Tomato*. Razdan, M. K. and Mattoo, A. K., Eds. *Science Publishers, Enfield,* NH. pp. 379–419.

Yassin, A. M. and Mour, M. A. 1965. Tomato leaf curl disease in Sudan and their relation to tobacco leaf curl. *Ann. Appl. Biol*. **56**: 207-217.

Yoshimura, A., Yoshimura, N., Iwata, S. R., McCouch, M. C., Abenes, M. R., Baraoidan, T. W., Mew, R. and Nelson, J. 1995. Tagging and combining ability of bacterial blight resistance genes in rice using RAPD and AFLP markers. *Mol. Bred*. **1**: 375-387.

Young, N. D. and Tanksley, S. D. 1989. RFLP analysis of the size of chromosomal segments retained around the *Tm-2* locus of tomato during backcross breeding. *Theor. Appl. Genet.* **77**: 353-359.

Yu, Z. H., Mackill, D. J., Bonman, J. M. and Tanksley, S. D. 1991. Tagging genes for blast resistance in rice via linkage to RFLPmarkers. *Theor. Appl. Genet.* **81**: 471-476.

Yuanfu, J. J., Scott, W. and Schuster, D. J. 2009. Molecular mapping of *Ty-4*, a new tomato yellow leaf curl virus resistance locus on chromosome 3 of Tomato. *J. Amer. Soc. Hort. Sci.* **134 (2)**: 281-288.

Zamir, D., Eksteinmichelson, I., Zakay, Y., Navot, N., Zeidan, M., Sarfatti, M., Eshed, Y., Harel, E., Pleban, T., Vanoss, H., *et al*., 1994. Mapping and

introgression of a tomato yellow leaf curl virus tolerance gene, *Ty-1*. *Theor. Appl. Genet.* **88**: 141–146.

Zamir, D., Tranksley, S. D. and Jones, R. A. 1982. Haploid selection for low temperature tolerance of tomato pollen. *Genetics* **101**: 129-137.

Zane, L., Bargelloni, L. and Patarnello, T. 2002. Strategies for microsatellite isolation: a review. *Mol. Ecol.* **11**: 1-16.

Zhang, G., Angeles, E. R., Abenes, M. L. P., Khush, G. S. and Huang, N. 1994. Molecular mapping of a bacterial blight resistance gene on chromosome 8 in rice. *Rice Genet. Newl.* **11**: 142-144.

Zietkiewicz, E., Rafalski, A. and Labuda, D. 1994. Genomic fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* **20**:176-183.

ANNEXURE I

Reagents required for DNA isolation

Reagents:

1. 2 X CTAB extraction buffer (100 ml)

CTAB	:	2g
(Cetyl trimethyl ammonium bromide)		
Tris HCl	:	1.21 g
EDTA	:	0.745 g
NaCl	:	8.18 g
PVP	:	1.0 g

Adjusted the pH to 8 and made up final volume up to 100 ml.

2. CTAB (10 %, 100 ml)

CTAB	:	10 g
NaCl	:	4.09 g

3. Chloroform- Isoamyl alcohol (24:1 v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

4. Chilled isopropanol

Isopropanol was stored in refrigerator at 0ºC and was used for the study.

5. Ethanol (70 %)

To the 70 parts of absolute ethanol (100 %), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. TE buffer (pH 8, 100 ml)

Tris HCl (10 mM)	:	0.1576 g
EDTA (1 mM)	:	0.0372 g

The solution was prepared, autoclaved and stored at room temperature.

ANNEXURE II

Composition of buffers and dyes used for gel electrophoresis

1. TAE Buffer 50 X

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5 M EDTA (pH 8.0)	:	100 ml

2. Loading Dye (6 X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in a dark bottle.

ANNEXURE III

Scoring of F_3 population with respect to the polymorphic band obtained using the primers (HB 12, LEaat 16, $TSCAR_{AAG/CAT}$ and Ualty 16)

Sl. No.	Plant number	Field reaction		Polymorphism observed in F_3 population			
				ISSR	SSR	SCAR	
		BW	ToLCV	HB 12	LEaat 16	$TSCAR_{AAG/CAT}$	Ualty 16
F_2-06							
01	F2-06-01	R	S	0	1	1	1
02	F2-06-02	R	R	1	1	1	1
03	F2-06-03	S		0	1	1	1
04	F2-06-04	R	S	0	1	1	1
05	F2-06-05	R	S	0	1	1	0
06	F2-06-06	R	S	0	1	1	0
07	F2-06-07	S		0	1	1	0
08	F2-06-08	R	S	0	1	1	1
09	F2-06-09	R	S	0	1	1	1
10	F2-06-10	R	S	0	1	1	0
11	F2-06-11	R	S	0	1	1	1
12	F2-06-12	R	S	0	1	1	1
13	F2-06-13	S		0	0	0	0
14	F2-06-14	S		0	0	1	0
15	F2-06-15	R	S	0	1	1	1
16	F2-06-16	R	S	0	1	1	1
17	F2-06-17	S		0	1	1	0
18	F2-06-18	S		0	0	0	1
19	F2-06-19	S		0	1	1	0
20	F2-06-20	R	S	0	1	1	1
21	F2-06-21	S		0	0	0	0
22	F2-06-22	R	R	1	1	1	1
23	F2-06-23	S		0	0	1	0
24	F2-06-24	R	S	0	1	1	0
25	F2-06-25	R	S	1	1	1	0
26	F2-06-26	R	S	1	1	1	1
27	F2-06-27	R	S	0	1	1	1
28	F2-06-28	R	S	0	1	1	1
29	F2-06-29	R	S	0	1	1	0
30	F2-06-30	R	S	0	1	1	1
31	F2-06-31	S		0	0	0	1
32	F2-06-32	S		0	1	1	1
33	F2-06-33	S		0	1	1	0
34	F2-06-34	S		0	0	1	0
35	F2-06-35	S		0	1	1	0
36	F2-06-36	S		0	1	1	1
37	F2-06-37	S		0	1	1	0
38	F2-06-38	R	S	0	1	1	0
39	F2-06-39	R	S	0	1	1	0
40	F2-06-40	S		0	1	0	0
41	F2-06-41	S		0	1	0	0

contd…

Sl. No.	Plant number	Field reaction		ISSR	SSR	SCAR	
		BW	ToLCV	HB 12	LEaat 16	TSCAR AAG/CAT	Ualty 16
42	F2-06-42	S		0	0	0	0
43	F2-06-43	S		0	1	1	0
44	F2-06-44	S		0	0	0	0
45	F2-06-45	R	S	0	0	1	0
46	F2-06-46	S		0	0	0	0
47	F2-06-47	R	S	0	1	1	0
48	F2-06-48	S		0	1	0	0
49	F2-06-49	S		0	1	0	0
50	F2-06-50	R	S	0	1	1	0
F_2-18							
51	F2-18-01	R	S	0	1	1	0
52	F2-18-02	S		1	1	1	1
53	F2-18-03	S		0	1	0	1
54	F2-18-04	S		0	0	1	0
55	F2-18-05	S		0	1	0	1
56	F2-18-06	R	S	0	1	1	1
57	F2-18-07	S		0	0	0	1
58	F2-18-08	R	R	1	0	1	0
59	F2-18-09	S		0	1	0	0
60	F2-18-10	R	S	0	1	1	0
61	F2-18-11	R	S	0	1	1	0
62	F2-18-12	S		1	1	1	0
63	F2-18-13	S		0	0	0	0
64	F2-18-14	R	R	1	0	1	0
65	F2-18-15	S		0	1	0	0
66	F2-18-16	S		0	1	0	1
67	F2-18-17	S		0	1	1	1
68	F2-18-18	R	S	1	0	1	0
69	F2-18-19	R	S	0	1	1	1
70	F2-18-20	S		0	1	1	0
71	F2-18-21	R	S	0	1	1	0
72	F2-18-22	R	R	1	1	1	1
73	F2-18-23	S		0	0	0	0
74	F2-18-24	R	S	0	1	1	1
75	F2-18-25	R	S	1	0	1	1
76	F2-18-26	S		0	0	0	0
77	F2-18-27	R	S	0	1	1	1
78	F2-18-28	R	S	1	1	1	1
79	F2-18-29	S		1	1	0	0
80	F2-18-30	R	S	1	1	1	1
81	F2-18-31	R	S	0	1	1	0
82	F2-18-32	R	S	1	1	1	0
83	F2-18-33	S		0	1	0	0
84	F2-18-34	R	S	0	1	1	0

contd…

Sl. No.	Plant number	Field reaction		ISSR	SSR	SCAR	
		BW	ToLCV	HB 12	LEaat 16	TSCAR AAG/CAT	Ualty 16
85	F2-18-35	R	S	1	1	1	0
86	F2-18-36	R	S	1	1	1	0
87	F2-18-37	R	S	0	1	1	0
88	F2-18-38	R	S	0	1	1	1
89	F2-18-39	R	R	1	1	1	0
90	F2-18-40	R	S	1	0	1	0
91	F2-18-41	S		0	0	0	1
92	F2-18-42	R	S	0	1	1	0
93	F2-18-43	S		1	1	0	1
94	F2-18-44	S		0	0	0	0
95	F2-18-45	R	S	1	1	0	0
96	F2-18-46	S		0	0	0	0
97	F2-18-47	S		0	0	0	0
98	F2-18-48	S		1	0	0	0
99	F2-18-49	R	S	0	1	1	0
100	F2-18-50	R	S	0	1	1	0
F_2-20							
101	F2-20-01	R	S	0	1	1	0
102	F2-20-02	R	S	0	1	1	0
103	F2-20-03	R	S	0	0	0	0
104	F2-20-04	R	S	0	1	1	0
105	F2-20-05	R	R	0	1	1	0
106	F2-20-06	R	S	0	1	0	0
107	F2-20-07	R	S	0	1	1	0
108	F2-20-08	S		0	1	0	0
109	F2-20-09	S		0	0	0	0
110	F2-20-10	S		0	0	0	0
111	F2-20-11	S		0	0	0	0
112	F2-20-12	S		0	0	0	0
113	F2-20-13	R	S	0	1	1	0
114	F2-20-14	S		0	0	0	0
115	F2-20-15	S		0	0	0	0
116	F2-20-16	S		0	0	0	0
117	F2-20-17	S		0	0	0	0
118	F2-20-18	S		0	0	0	0
119	F2-20-19	R	S	0	1	1	0
120	F2-20-20	S		0	0	0	0
121	F2-20-21	R	S	0	1	1	0
122	F2-20-22	R	S	0	1	1	0
123	F2-20-23	R	S	0	1	1	0
124	F2-20-24	R	S	0	1	0	0
125	F2-20-25	S		0	1	0	0
126	F2-20-26	R	S	0	0	1	0
127	F2-20-27	S		0	0	0	0

contd…

Sl. No.	Plant number	Field reaction		ISSR	SSR	SCAR	
		BW	ToLCV	HB 12	LEaat 16	TSCAR AAG/CAT	Ualty 16
128	F2-20-28	S		0	1	0	0
129	F2-20-29	R	S	0	1	1	0
130	F2-20-30	R	S	0	0	0	0
131	F2-20-31	S		0	0	0	0
132	F2-20-32	R	S	0	1	0	0
133	F2-20-33	R	S	0	1	1	0
134	F2-20-34	R	S	0	1	1	0
135	F2-20-35	S		0	1	0	0
136	F2-20-36	S		0	0	0	0
137	F2-20-37	S		0	1	0	0
138	F2-20-38	S		0	0	0	0
139	F2-20-39	S		0	0	0	0
140	F2-20-40	R	R	0	1	0	0
141	F2-20-41	S		0	0	0	0
142	F2-20-42	S		0	0	0	0
143	F2-20-43	S		0	0	0	0
144	F2-20-44	S		0	0	0	0
145	F2-20-45	S		0	0	0	0
146	F2-20-46	S		0	0	0	0
147	F2-20-47	S		0	0	0	0
148	F2-20-48	S		0	0	0	0
149	F2-20-49	S		0	1	0	0
150	F2-20-50	S		0	0	0	0
F_2-33							
151	F2-33-01	S		1	0	0	0
152	F2-33-02	R	S	1	1	1	0
153	F2-33-03	R	S	1	1	1	0
154	F2-33-04	S		0	1	0	0
155	F2-33-05	S		1	1	0	0
156	F2-33-06	S		1	0	1	0
157	F2-33-07	S		1	0	0	0
158	F2-33-08	R	S	1	1	1	0
159	F2-33-09	R	S	1	1	1	0
160	F2-33-10	S		1	1	1	0
161	F2-33-11	S		1	0	0	0
162	F2-33-12	R	S	1	1	1	0
163	F2-33-13	S		1	1	0	0
164	F2-33-14	S		1	1	1	0
165	F2-33-15	S		1	1	1	0
166	F2-33-16	S		1	1	0	0
167	F2-33-17	R	S	1	1	1	1
168	F2-33-18	S		1	0	0	1
169	F2-33-19	S		1	1	1	1
170	F2-33-20	S		1	1	1	1

contd…

Sl. No.	Plant number	Field reaction		ISSR	SSR	SCAR	
		BW	ToLCV	HB 12	LEaat 16	TSCAR AAG/CAT	Ualty 16
171	F2-33-21	R	S	1	1	1	0
172	F2-33-22	R	S	1	1	1	1
173	F2-33-23	R	R	1	1	1	0
174	F2-33-24	R	S	1	1	1	0
175	F2-33-25	S		1	0	0	0
176	F2-33-26	S		1	0	0	0
177	F2-33-27	R	S	1	1	1	1
178	F2-33-28	S		0	0	0	1
179	F2-33-29	S		1	1	1	0
180	F2-33-30	R	R	1	1	1	1
181	F2-33-31	R	S	0	1	1	1
182	F2-33-32	R	S	1	1	1	1
183	F2-33-33	S		1	0	0	0
184	F2-33-34	S		1	0	0	0
185	F2-33-35	R	S	1	1	0	0
186	F2-33-36	S		1	0	1	0
187	F2-33-37	R	R	1	1	0	1
188	F2-33-38	R	R	1	1	1	0
189	F2-33-39	S		1	0	0	0
190	F2-33-40	R	S	1	1	1	0
191	F2-33-41	S		0	0	0	0
192	F2-33-42	R	S	1	0	1	0
193	F2-33-43	S		1	0	0	0
194	F2-33-44	S		1	1	0	1
195	F2-33-45	R	S	1	1	1	0
196	F2-33-46	R	S	1	1	1	0
197	F2-33-47	S		1	0	0	0
198	F2-33-48	S		1	1	0	1
199	F2-33-49	S		0	0	0	0
200	F2-33-50	R	S	0	1	1	0

BW- Bacterial wilt, ToLCV- Tomato leaf curl virus, R- Resistance, S- Susceptible, 1- Polymorphic band present, 0- Polymorphic band absent.

INTEGRATION OF COMBINED DISEASE RESISTANCE FOR BACTERIAL WILT AND ToLCV IN TOMATO (*Solanum lycopersicum* L.) THROUGH MARKER ASSISTED SELECTION

By

BELGE SHRIRAM ASHRU

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ABSTRACT OF THE THESIS

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Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2014

ABSTRACT

Tomato (*Solanum lycopersicum* L.) is one of the most consumed vegetables after potato in the world and is an excellent plant genetic analysis system. It is grown in 0.88 million hectares area with 20.7 metric tonnes productivity in India (www.nbh.gov.in).

The area under tomato cultivation in Kerala is very meagre. The main limiting factor is the incidence of bacterial wilt caused by *Ralstonia solanacearum* and ToLCV infection. The warm humid tropical climate and acidic soil conditions favor the incidence of this disease in Kerala. Crop loss upto 100 per cent is reported due to bacterial wilt. ToLCV affected plants exhibit curling, puckering, reduction in leaflet size, severe stunting and reduction in fruit set. However, severely infected plants almost fail to produce fruits.

Traditional breeding at KAU has resulted in development of bacterial wilt resistant varieties (Sakthi, Mukthi and Anagha) which are susceptible to ToLCV. Genotypes resistant to different strains of ToLCV have been developed at Indian Institute of Horticulture Research. Genotypes with combined resistance and better agronomic traits are greatly warranted to improve tomato cultivation in problem areas. Molecular markers have been used extensively for genome mapping as well as identification and characterization of genes and QTL for many agriculturally important traits in tomato; including disease resistance. When sources of resistance to bacterial wilt (Sakthi) and tomato leaf curl virus (IIHR 2196) are available, these two characters can be combined in a single genotype with the help of marker assisted selection. Keeping this as the crucial aim, the present investigation was undertaken during 2013-2014 for screening of F_3 population of a cross between Sakthi (BW resistant; low yield) and IIHR 2196 (ToLCV resistance; high yield) so as to integrate combined disease resistance along with better horticultural traits.

Four mother plants selected from F_2 population used for the study included F_2-06, F_2-18, F_2-20 and F_2-33 having both bacterial wilt and ToLCV resistance. Sixty seeds from each mother plant were used for raising F_3 population. Two hundred F_3 plants were evaluated for bacterial wilt and ToLCV disease reaction and important biometric parameters were recorded. Molecular markers reported earlier for bacterial wilt and ToLCV were validated on parents and F_3 population.

DNA isolated from parents Sakthi and IIHR 2196 were used to validate 14 ISSR, 14 SSR and 8 SCAR primers already reported for BW and ToLCV resistance. One ISSR, 3 SSR and 2 SCAR primers which showed polymorphism and reproducibility among parents were selected for F_3 population screening.

Biometric characters of F_3 population raised in disease sick field were recorded along with disease reaction for bacterial wilt and ToLCV. Analysis of variance indicated superiority for F_2-33 (1347.27 g) with respect to yield per plant. In F_3 population 12 plants showed combined resistance for bacterial wilt and ToLCV and four of them were good yielders too (2575 to 3281 g/plant).

Among the six selected primers used to screen the segregating population, only two each were found specific for bacterial wilt and ToLCV. The SSR LEaat 16 and $TSCAR_{AAG/CAT}$ were identified for bacterial wilt and ISSR HB 12 and SCAR Ualty 16 for ToLCV. Since HB 12 is an ISSR marker, it has to be further validated before use in marker assisted selection. The other three primers selected can be recommended for marker assisted selection in tomato.