Development of a molecular marker for bacterial wilt resistance in brinjal (*Solanum melongena* L.) varieties Surya and Swetha

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Development of a molecular marker for bacterial wilt resistance in brinjal (*Solanum melongena* L.) varieties Surya and Swetha"** 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
27.08.2011

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CERTIFICATE

Certified that this thesis entitled " **Development of a molecular marker for bacterial wilt resistance in brinjal (*Solanum melongena* L.) varieties Surya and Swetha"** is a bonafide record of research work done independently by Ms. Somya P.P(2009-11-122) under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Somya P.P

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ABBREVIATIONS

bp	Base pairs
β	Beta
cm	Centimeter
CPBMB	Centre for Plant Biotechnology and Molecular biology
CTAB	Cetyl Trimethyl Ammonium Bromide
^0C	Degree Celsius
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
Kb	Kilo basepairs
KAU	Kerala Agricultural university
L	Litre
mA	Milli Ampere
Mb	Mega base pairs
Min	Minutes
ml	Millilitre
µg	Microgram
µl	Microlitre
ng	Nanogram
OD	Optical Density
pH	Hydrogen ion concentration
PCR	Polymerase Chain Reaction
PVP	Poly vinyl pyrolidine
%	Percentage
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic acid
RNase	Ribonuclease
RP	Resistant Parent
rpm	Rotations per minute
SCAR	Sequence Characterized Amplified Region
Sec	Second (s)
SP	Susceptible Parent
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra violet
V	Volts
v/v	Volume by Volume
w/v	Weight by Volume

Introduction

1.INTRODUCTION

The brinjal (*Solanum melongena* L.) known as egg plant or guinea squash is a member of the Solanaceae family grown extensively in central, southern and southeast Asia and in a number of African countries (Kalloo, 1993). The plant is native to India. Globally it is grown on 1957 thousand hectares, with a total production of 10378 thousand tones. The leading brinjal producers are China (56% of world output), India (26% of world ouput), Egypt and Turkey.

Brinjal occupies the third position amongst vegetable crops grown in India. It contributes 9% of the total vegetable production of the country. West Bengal is the largest producer of brinjal followed by Maharashtra and Bihar. The other main states growing brinjal are Karnataka, Maharashtra, Gujarat, Andhra Pradesh, Assam and Madhya Pradesh. Brinjal, chilli and tomato are the important solanaceous fruit vegetables grown in Kerala. Many pests and diseases which infect other solanaceous vegetables, such as tomato, pepper (capsicum), and potato, are also troublesome to brinjal.

Bacterial wilt caused by *Ralstonia solanacearum* (Smith, 1896) Yabuuchi *et al.,* 1995 is one of the most serious diseases of crops in tropics, subtropics and warm temperate regions of the world. Losses due to wilt is between 10.8 to 90.6 per cent depending on the stage of the crop and environmental condition. In India 75-81 per cent loss is reported in brinjal due to bacterial wilt (Singh, 1995). In Kerala, the disease is widespread in solanaceous vegetables and ginger. The warm, humid tropic and acidic soil condition of Kerala makes it a hot spot for wilt incidence.

Management strategy of bacterial wilt disease caused by *R. solanacearum* involves spraying of antibiotics like Agrimycin-100, Chloramphenicol or Streptomycin. But these chemicals have no effect if applied after infection. Applications of chemical pesticides are not only harmful to human health and the

environment, but are not effective in controlling the disease especially during favourable conditions. Hence use of resistant varieties are preferred for disease management.

The Department of Olericulture, College of Horticulture has developed bacterial wilt resistant varieties like Surya, Swetha, Haritha and Neelima in brinjal (Gokulapalan *et al.*, 2004). Surya (SM 6-7) with purple, oval fruits is a national variety. It was developed by single plant selection from an Annamalai collection SM 6. Swetha (SM 6-6) with white elongated, small fruits was developed from SM 6 by single plant selection. Varghese (1991) found that inheritance of bacterial wilt resistance in these varieties is monogenic and homozygous recessive. The resistance available in these varieties can be used for imparting disease resistance in other superior brinjal varieties.

Developments in biotechnology have led plant breeders to develop more efficient selection systems to replace traditional phenotypic-pedigree-based selection systems. DNA marker technology has been used in commercial plant breeding since early 1990's. Marker assisted selection (MAS) is an indirect selection process where a trait of interest is selected not based on the trait itself but on a molecular marker linked to it. The assumption is that linked allele associates with the gene or quantitative trait locus (QTL) of interest. Some of the commonly used molecular markers for genotype identification are RAPD, ISSR, RFLP, AFLP and SSR.

Markers remove the impact of environmental variation, which often complicates phenotypic evaluation. MAS can be useful for traits that are difficult to measure, exhibit low heritability, and are expressed late in development. Once traits have been mapped and a closely linked marker has been found, it is possible to screen large numbers of samples for rapid identification of progeny that carry desirable characteristics; also genetic quality is vastly increased because screening is carried out at an early stage. Markers linked to the resistance gene may also be useful for cloning and sequencing, the gene of interest.

Bulked segregant analysis is a breeding method reported by Michelmore *et al.* (1991) for tagging a gene of interest, which involves comparing two pooled DNA samples of individuals from a segregating population originating from a single cross. Within each pool or bulk, the individuals are identical for the trait or gene of interest but are arbitrary for all other genes. BSA removes the need for screening the entire mapping population with every primer. The minimum size of the bulk is determined by the frequency with which linked loci might be detected as polymorphic between the bulked samples.

The present study was proposed to develop a molecular marker for bacterial wilt resistance in brinjal varieties Surya and Swetha. The methodology involves bulk segregant analysis of single plant population of F_2 generation raised by crossing resistant variety Surya with a highly susceptible variety Pusa Purple Long using RAPD marker. The DNA of Swetha will be included in bulk segregant analysis because it is also a single plant selection from Annamalai collection.

The main objectives of the study were the following:

1. To develop a molecular marker for bacterial wilt resistance gene in brinjal variety Surya with RAPD analysis
2. To test the suitability of the same for identifying bacterial wilt resistance trait of resistant variety Swetha

Review of literature

2. REVIEW OF LITERATURE

The research programme entitled "Development of a molecular marker for bacterial wilt resistance in brinjal (*Solanum melongena* L.) varieties Surya and Swetha" was selected for developing a molecular marker for identifying these varieties at DNA level. The relevant literatures on various aspects of the investigation is reviewed in this chapter.

2.1. BACTERIAL WILT

Bacterial wilt is a limiting factor for brinjal cultivation in tropical, subtropical and some warm temperate climates (Sunaina and Tomar, 2003). Losses due to wilt is between 10.8 to 90.6 per cent depending on the stage of crop and environmental condition. More loss reported in summer season when crop gets infected within 60 days of planting. Over one hundred years have elapsed since Erwin F. Smith published the first description of *Pseudomonas solanacearum* E. F. Smith that causes wilt disease of solanaceous plants (Smith, 1896).

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

2.1.1. Symptoms

The initial symptoms of the disease on brinjal are sudden wilting of youngest leaves which may recover during night. With the progress of the disease, initial wilt is succeeded by a sudden and permanent wilting of entire plant with a slight or no leaf yellowing. Infected young plants die immedietly while older

plants may first show leaf drooping and discoloration only on one side or part of the plant and eventually the whole plant wilt and die. The bacterium colonises the xylem vessels and causes vascular plugging. The vascular system in affected plants is discoloured from pale yellow to brown (Gupta and Thind, 2006).

2.1.2. The pathogen

The disease is caused by the bacterium *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* It is a gram-negative, plant pathogenic, aerobic, non-sporing, rod, motile soil bacterium with a polar flagellar tuft. Strains of this pathogen differ in host range, geographical distribution, pathogenicity and physiological properties. Strains are subdivided informally into 5 races based upon host range. Buddenhagen *et al.* (1962) classified *R. solanacearum* isolates from a wide host range in Central and South America based on host range, pathogenicity and colony appearance on Triphenyl Tetrazolium Chloride (TZC) medium, into 3 races *viz.*, race 1, race 2, race 3. Race 4 (Aragaki and Quinon, 1965) and race 5 (He *et al.*, 1983) also were reported.

1. Race 1 (Solanaceous strain) - It has a wide host range and is seen distributed throughout the lowlands of tropics and subtropics. They attack tomato, tobacco, solanaceous crops and other weeds
2. Race 2 (Musaceous strain) - Host range include a *Musa* spp. and a few perennial hosts. This race was restricted to American tropics and few parts of Asia
3. Race 3 (Potato strain) - Host range includes potato and few alternate hosts in tropics and subtropics
4. Race 4 - Host is ornamental ginger
5. Race 5 - Host is mulberry

Hayward (1964) classified *R. solanacearum* into biotypes or biochemical types or biovars namely biovar I, biovar II, biovar III, and biovar IV based on their ability to oxidise various carbon sources (three disaccharides and three sugar alcohols) and on other bacteriological reactions.

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

The prevalent races in India are race 1, 3, 4 and biovar 11,111,1V (Mathew *et al.*, 2000; Sunaina and Tomar, 2003). After studying different isolates from many parts of Kerala, Remadevi (1978) reported that *R. solanacearum* exists in different races or strains coming under race 1 or race 3. Mathew (2001) and Mathew *et al.* (2002) reported biovar 3, 3A and 5 of tomato isolates of *R.solanacearum* from different locations of Thrissur and Palakkad districts of Kerala.

2.1.3. Host range

Bacterial wilt affects more than 200 plant species belonging to over 40 families. Important hosts are potato, pepper, tobacco, tomato, eggplant and banana (Bulbul *et al.*, 2001). *Ralstonia solanacearum* could survive in the rhizosphere, on the rhizoplane and inside the roots of a large number of weed plants like *S. nigrum, S. anguivi, Euphorbia hirta, Colocasia esculenta* (Samaddar *et al.*, 1998).

2.1.4. Disease Cycle

The bacterium can survive in moist soil for longer periods in the absence of plant debris. Diseased plant debris also helps in overseasoning of the bacterium. The pathogen can be seed borne (Sumitra *et al.*, 2000) or can survive for long periods on surface water (Mazzucchi *et al.*, 2000). Entry of pathogen in the host via root occurs through mechanical/nematode/ insect injury or growth cracks. Enhancement in the development of symptoms is seen in the presence of root knot nematodes (Verma *et al.*, 1997). The bacterium colonises the xylem vessels and spreads throughout the plant. The incubation period varies depending upon age and susceptibility of the plant involved. The bacteria invade the intercellular spaces of the parenchyma cells in the cortex and pith and disintegrate

the cell wall resulting in pockets filled with bacterial and polysaccharide and cellular debris. Bacteria are released back into the soil from infected roots and decaying plant material (Bruehl, 1987).

2.1.5. Epidemiology

The bacterium survives from one season to another in infected plant debris in soil, in seed, or surface water besides wide host range. The pathogen doesn't survive and reproduce below 10°C (vanElsas *et al.*, 2000) although it can survive over a wide range of temperatures ie. 15-37°C and optimum of 35-37°C. The disease usually develops when mean temperature is above 20°C and more severe wilting is seen at higher temperatures of 30°C or more and higher soil moisture. The pathogen can grow over a wide range of pH from 6.2-7.4 but alkaline soils are generally unfavourable for disease development. Dissemination of bacteria occurs with irrigation or rain water, on infected plants, infected seeds and soil (Goto, 1999).

2.1.6. Management

No chemical has been found effective against the disease once the infestation has occurred. Nematodes in the soil should be eradicated with the use of nematicides because in their presence symptoms are expressed earlier than alone and incidence of the disease increases. Cultural practices like use of healthy seed, suitable crop rotation, control of weeds and volunteer plants, avoidance of surface water for irrigation (Baginska and Kordyla-Bronka, 1998), avoidance of movement of soil from infected fields to disease free fields through equipment and workers, and disinfestations of the equipment should be followed to keep the disease under check. Nursery should be grown in disease free soil and should be disinfected before sowing either with soil solarisation on steam sterilized or treatment with formaldehyde.

2.2. ARTIFICIAL INOCULATION

Winstead and Kelman (1952) tried four methods of artificial inoculations in tomato like (1) stem puncturing at the third node below the apex (2) cutting the lateral roots along one side and pouring bacterial suspension over soil (3) pouring bacterial suspension over soil without injury to the roots and (4) dipping roots in bacterial suspension. They found that symptom of wilt became first evident by stem puncturing at third node below apex. Using this method at the end of 15 days all plants were dead.

Pradhanang *et al.* (2000) inoculated *Drymaria cordata* and *Polygonus capitata* with *R. solanacearum* biovar 2 by root injury. The roots were cut by inserting a sterile scalpel into the soil to one side of the plant to aid infection. Within two months of infection all plants were killed.

Janse *et al.* (2004) inoculated *R. solanacearum* biovar 2 Race 3 in *Pelargonium zonale* cuttings by 1) microinjection of 10µl inoculums into the lower stem 2) pouring 35ml bacterial suspension (10^7 cfu ml^{-1}) per plant in soil around un-injured plants. Microinjection was found to be effective and symptoms appeared within seven days.

Resmi *et al.* (2009) screened somaclones of ginger (*Zingiber officinale*) for bacterial wilt disease by 1) planting in sick field 2) inducing electrolyte leakage using toxic metabolite pathogen 3) artificial inoculation by placing a piece of cotton dipped in bacterial ooze in pin pricked area on second leaf axil. First and third method was found to be very effective and symptoms appeared within seven days.

Bi-hao *et al.* (2009) inoculated seedlings at 5-6 leaf stage with *R. solanacearum* inoculums. Plant roots were wounded before inoculation and roots

were put in bacterial suspension for half an hour. Symptoms appeared within 10 days and within two months entire plants were dead.

2.3. DEFENCE MECHANISM OF PLANTS

2.3.1. Genetic control of resistance

Resistance to bacterial wilt is inherited in a recessive and monogenic manner in solanaceous crops (Gopimony, 1983; Varghese, 1991). Geetha and Peter (1993) reported that F_1 hybrids in which only resistant parents were involved were resistant, while the hybrids in which susceptible genotype was one of the parents were either susceptible or moderately resistant showing recessive mode of inheritance of bacterial wilt resistance in brinjal. Studies conducted at Kerala Agricultural University, Vellanikkara in brinjal and chilli revealed that resistant F_1s could be developed by crossing resistant parents which indicates recessive mode of inheritance of bacterial wilt resistance (KAU, 1989).

2.3.1.1. Resistance genes (R genes) and QTL

Plants have developed a wide array of defense responses to control pathogen invasion. Among these the presence or absence of complimentary pair of resistance (*R*) genes in the host and avirulence gene (*avr*) in the invading microorganisms determines the outcome of many plant pathogen interactions.

In the elicitor –receptor model proposed to account for the gene-for-gene theory (Flor, 1971), *avr* genes encode elicitors that serve as ligands for receptors encoded by *R* genes which trigger a complex defense response. When the genes for resistance in host match with the genes for avirulence in pathogen the cultivar will be resistant; otherwise resistance will break down.

Most of the genes contain a leucine-rich repeat (LRR) domain, which function in mediating protein-protein interactions. Several cloned R genes belong to the nucleotide binding site (NBS)-LRR class (Staskawicz, 2001). Many of the *R* gene products share structural motifs which indicate that resistance to diverse pathogens may operate through similar pathways. The use of molecular markers and interval mapping is a powerful approach for identification and mapping of loci controlling trait of interest. Several QTL's have shown to play a role in resistance to bacterial wilt in different studies (Danesh *et al.*, 1994).

Jones *et al.* (1994) reported class of R genes that encode extracellular LRR's with a transmembrane domain (ie. receptor like proteins RLP's) in tomato conferring resistance to *Cladosporium fulvum.* Xiao *et al.* (2003) found that powdery mildew resistance gene *RPW 8* in *Arabidopsis thaliana* confers broad spectrum of resistance. It is said to be having an amino-terminal transmembrane domain and a CC domain. Belfanti *et al.* (2004) reported the *Hcrvf2* gene from a wild apple cultivar which confers scrab resistance to a transgenic cultivated variety.

Ashfield *et al.* (2004) cloned *Rpg -1b* from soybean and compared that gene to the functionally analogous CNL gene, *RPM1* of *Arabidopsis thaliana.* Both genes confer resistance to *Pseudomonas syringae* expressing avrB. The level of amino-acid sequence identity between the encoded *RPM1* and *Rpg-1b* protein was found to be low and they were not orthologous; because both plant genes recognize the *avrB* specificity, these genes must have independently evolved nearly identical functions.

Witsenboehr *et al.* (1995) reported that at particular genomic regions, *R* genes conferring resistance to viral, bacterial and fungal pathogens are loosely clustered approximately 1-2 cm apart in Arabidopsis, lettuce and soyabean. Deberdta *et al.* (1999) reported *Mi* genes associated with bacterial wilt resistance in tomato lines Caraibo, CRA66 and Cranita.

Deslandes *et al.* (2003) identified and mapped *RRS1*, a single recessive locus in *Arabidopsis thaliana* (Nd-1 ecotype) that confers resistance to *R. solanacearum*. *RRS1-R* is the first characterized *R* gene conferring resistance to *R. solanacearum* and its characterization, the elucidation of its mode of actions as well as its intergeneric transfer in various crops are important steps in facilitating the elaboration of new disease control approaches.

2.3.1.2 .Classification of Resistant genes

There are several classes of resistant genes. The major classes are the NBS-LRR genes, the cell surface pattern recognition receptors (PRR), extracellular LRR class of R genes and the Pto gene.

1) NBS-LRR genes

NBS-LRR genes contain a nucleotide binding site (NBS) and a leucine rich repeat (LRR). The protein products encoded by this class of genes are located within the plant cell cytoplasm.

There are two subclasses with in NBS-LRR class of R genes

a) One terminal has an amino-terminal/Interleukin 1 receptor homology region (TIR)

b) The other subclass contain a leucine zipper region in its amino-terminal (McHale *et al.*, 2006)

2) PRR genes

The protein product of PRR's contain extracellular, juxtamembrane, transmembrane and intracellular non-RD kinase domain. Lee *et al.* (2009) reported that PRR class of R genes include rice *XA21* resistance gene that recognize *ax21* peptide and the Arabidopsis *FLS2* peptide that recognizes the *flg22* peptide from flagellin (Wang *et al.*, 1995; 1998).

3) Extracellular LRR class of R genes

This group include rice *Xa21D* for resistance against Xanthomonas and the *cf* genes of tomato that confer resistance against *Cladosporium fulvum* (Lee *et al.*, *2009).*

4) Pto gene

The *Pseudomonas* tomato resistance gene (*Pto*) belongs to a class of its own. It encodes a Ser/Thr kinase but has no LRR. It requires the presence of a linked NBS-LRR gene, *prf* for activity (Wang *et al.*, 1998).

2.3.2. Biochemical and nutritional bases of resistance

Paul (1998) reported that resistant genotype had a higher content of OD phenol, polyphenol oxidase activity and alkaloids under healthy condition; and higher total phenol, soluble protein, specific activity and peroxidase activity under diseased condition. The higher phenol content may be responsible for preventing the entry of the pathogen and further multiplication in the roots of resistant varieties.

2.3.3. Anatomical basis of resistance

Singh (1996) observed large sized, less healthy and loosely arranged cortical cells in the roots of susceptible variety. The cortical cells of resistant ones were small sized and compactly arranged. This revealed the possibility of easy entry of pathogenic bacteria through the loosely arranged cortical cells of the susceptible variety. In the resistant variety, the compactly arranged cortical cells provide a shield against the entry of bacteria into the roots from the soil.

2.3.4. Products conferring resistance in hosts

1. Phenolics

Several kinds of phenolic compounds occur naturally in plants. They are antifungal, antibacterial and antiviral compounds. They inhibit toxin production, fungal spore germination, mycelia growth (Chet e*t al.*, 1978). Host enzymes like polyphenol oxidase and peroxidase oxidize phenolics to quinines which are more toxic.

2. Phytoalexins

Phytoalexins are low molecular weight, antimicrobial compounds that are synthesized and accumulated in plants after exposure to microorganism (Paxton, 1981). Phytoalexins are mostly isoflavanoids, terpenoids and polyacetylene compounds and synthesized *de novo* on infection by the pathogen. Elnaghy and Heitefuss (1976) detected phytoalexins in resistant bean varieties on inoculation with *Uromyces phaseoli*. Tjamos and Smith (1974) reported that tomato varieties resistant to *Verticillium albo-atrum* accumulated phytoalexins than susceptible varieties when inoculated with pathogen. Modolo *et al.* (2002) reported that soybean produce glyceollins, a phytoalexin as a defense mechanism in response to *Diaportha phaseolorum* f.sp.meridionalis elicitor.

3. Sugars

Sugars are pre-cursors for synthesis of phenolics, phytoalexins, lignin and callose. Hence they have an important role in defence mechanism of host.

4. Aminoacids

Aminoacids are the building blocks for the synthesis of proteins, and some phenolics, phytoalexins and lignin. Burrell (1981) showed that methionine metabolism is an important factor in disease resistance against potato scab. Emmanouil and Wood (1981) reported that various aminoacids induced synthesis of defense chemicals in tomato against Verticillium wilt.

5. Minerals

Certain minerals play an important role in disease resistance. Vidyasekharan (1990) reported that iron reduced the virulence of *Botrytis cinera* and decreased the disease intensity in beans. Zinc reduced the occurrence of 'Kresek' caused by *Xanthomonas campestris (*Mew *et al., 1979).*

2.4. SOURCES OF RESISTANCE

Gopimony (1983) exposed F_1 seeds of the cross *S. melongena* var. *insanum* and Purple Giant to gamma radiation to enhance recombination of bacterial wilt resistance found in former and better yield in the later variety.

Narayanan (1984) selected three wilt resistant varieties *viz.*, Pusa Purple Cluster, SM-6 as female parent and SM 1-10 and three susceptible commercial varieties *viz.*, Black Beauty, Pusa Purple Long and Pusa Purple Round as male parent for hybridization. Nine cross combinations were produced and all hybrids were found to possess field resistance and were on par with resistant parents.

Jessykutty (1985) reported that SM 6-2 and SM 6-4 were highly resistant after screening under field conditions. Geetha (1989) found that F_1 hybrids of the crosses SM 6-6 and SM 132, and SM 6-2 and PPC were promising for yield and were resistant to bacterial wilt.

Varma (1995) found that progenies of the crosses SM 6-6 and SM 197, SM 132 and SM 262 and SM 141 and SM 262 were found to be resistant to wilt. Chaudhary and Sharma (2000) reported that brinjal genotypes Arka Keshav, Arka Neelkanth, Arka Nidhi and SM 6-6 were resistant to bacterial wilt.

Gopalakrishnan and Singh (2000) assessed 10 brinjal cultivars and their hybrids for their resistance to bacterial wilt and observed that genotypes except

Composite 2, SM 63, Surya x Composite, Arka Keshav x Composite 2 and Swetha x SM 63 were not infected by bacterial wilt.

Gokulapalan *et al.* (2004) reported that high resistance to the most virulent isolates of *R. solanacearum* was noticed in the variety Surya (SM 6-7). Bi-hao *et al.* (2004) reported that in brinjal F_1 progenies of the cross E-31 (highly resistant, round fruit, from Huizhou local variety, Guangdong province) and E-32 (highly susceptible, round fruit) and BC1 with resistant parent as recurrent parent were resistant to *R. solanacearum* (race1).

Hussain *et al.* (2005) screened 15 accessions of brinjal in sick beds previously inoculated with *R. solanacearum* and found that accession EG 203 showed resistance to bacterial wilt. Swaroop *et al.* (2000) reported that Arka Keshav, BB-60-C, 95-4 Round and CHES -309 were resistant to bacterial wilt caused by *R. solanacearum.*

Rashid *et al.* (2002) reported that brinjal line 'Mixture' was resistant to bacterial wilt caused by *R. solanacearum* and the lines BL-156 (11), LG Long 1 and SOO-14 were moderately resistant.

2.5. DNA ISOLATION IN SOLANACEOUS CROPS

Pehu *et al.* (2006) standardized DNA isolation from young brinjal leaves. They modified Rogers and Bendich (1994) method by increasing the CTAB concentration from 2 per cent to 10 per cent with 0.7 M NaCl. Demir *et al.* (2010) isolated DNA from young leaves of brinjal using modified CTAB method. Modification used by them was addition of 100µl β-mercaptoethanol.

Archak *et al.* (2002) isolated DNA from tomato cultivars using modified CTAB method. DNA was treated with bovine pancreatic RNase and extracted once each with phenol: chloroform (1:1) and chloroform: isoamyl alcohol (24:1).

Karumannil (2007) reported that protocol suggested by Rogers and Bendich (1994) was ideal for DNA isolation from tomato leaves. Yang *et al.* (2007) reported DNA extraction method for sunflower leaves. They modified CTAB method with addition of 1.42M NaCl and phenol: chloroform: isoamyl alcohol extraction step to remove polysaccharides effectively.

Mirashamsi *et al.* (2008) isolated genomic DNA from tomato hybrids based on modified Dellaporta method. Nouhi *et al.* (2008) isolated DNA from frozen leaves of sugarbeet *(Beta* vulgaris) using CTAB method. Ragina (2009) isolated DNA from tomato leaves using modiefied CTAB method. Modifications done were addition of 100 µl β-mercaptoethanol and changing the quantity of extraction buffer.

Rifty *et al.* (1998) isolated DNA from tobacco using the procedure of Kochert *et al.* (1991) with modification like suspending the grinded leaves in 150 ml cold extraction buffer and centrifuging at 400 x for 20 min at 4°C. After adding chloroform: isoamyl alcohol the sample was centrifuged at 3000 Xg for 30 mts to obtain a clear supernatant.

2.6. DNA ISOLATION FROM OTHER CROPS

Singh *et al.* (2006) used modified CTAB extraction protocol by Xu *et al.* (1994) to isolate genomic DNA from lyophilized young leaves of sorghum. Sharma *et al.* (2008) modified DNA extraction method in tuber crops. This method followed inactivation of protein contaminants by using CTAB/ proteinase K and precipitation of polysaccharides in presence of high concentration of salt. Poczai *et al.* (2008) isolated DNA from 7 subgenus of *Solanum* using Walbot and Warren method (1988).

Pamidimarri *et al.* (2009) isolated genomic DNA from *Jatropha curcas* using modified CTAB protocol. The modifications done by them were 1) use of

3.5 M NaCl in extraction buffer 2) Tris saturated phenol during purification (pH 8) 3) 80% ethanol and 2.0M NaCl during precipitation.

Asish *et al.* (2010) isolated DNA from leaves and dry fruit rind of *Garcinia* sp. with modified Doyle and Doyle protocol like usage of 4 % CTAB followed by 1.5% PVP and 0.3% mercaptoethanol. In case of fresh fruit rind they used 2 % CTAB.

Iqbal *et al.* (2010) isolated DNA from Jatropha leaves using CTAB method with slight modifications like incubating at 65 °C for 90 mts. After incubation the contents were emulsified with chloroform: isoamyl alcohol (24:1) for 5 mts by inversion. The contents were centrifuged at 8000rpm for 10 mts. The upper aqueous phase was transferred in a presterilised centrifuge tube and two times extracted with an equal volume of chloroform: octanol (24:1).

Ginwal *et al.* (2010) isolated genomic DNA from *Acorus calamus* L. using modified CTAB method. Modifications done were usage of higher concentrations of polyvinyl pyrolidone, addition of lithium chloride solution as well as additional washing steps of DNA pellets.

Ginwal *et al.* (2010) isolated DNA from *Asparagus racemosus* Willd using modified CTAB method. Modifications used by them were higher volume of extraction buffer, increasing incubation temperature, use of higher concentration of PVP, repetition of purification step with chloroform: isoamyl alcohol and washing of DNA pellet with buffer and 80 per cent ethanol.

2.7. MARKER ASSISTED SELECTION

Within the last twenty years, molecular biology has revolutionized conventional breeding techniques in all areas. Biochemical and Molecular techniques have shortened the duration of breeding programs. The use of

molecular markers in conventional breeding techniques has also improved the accuracy of heterosis breeding and helped breeders to produce strains with combined traits that were difficult before the advent of DNA technology.

2.7.1. Types of Markers

1) Morphological Markers

These are the traditional markers. Morphological mutant traits in a population are mapped and linkage to a desirable or undesirable trait is determined and indirect selection is carried out using the physically identifiable mutant for the trait (Singh, 2008).

2) Biochemical Markers

Isozymes are used as biochemical markers in plant breeding and genetics as neutral genetic markers (Markert and Moller, 1959). They are proteins produced by gene expression. These proteins are extracted, and run on denaturing electrophoresis gels. The denaturing component in the gels (usually SDS) unravels the secondary and tertiary structure of the enzymes and they are then separated on the basis of net charge and mass. Polymorphic differences occur on the amino acid level allowing singular peptide polymorphism to be detected and utilized as a polymorphic biochemical marker (Chawla, 2010).

3) Molecular Markers

Molecular markers are based on naturally occurring polymorphisms in DNA sequences (ie. base pair deletions, substitutions, additions). There are various methods to detect and amplify these polymorphisms so that they can be used for breeding analysis. Molecular markers are superior to other forms of MAS because they are relatively simple to detect, abundant throughout the genome even in highly bred cultivars, completely independent of environmental conditions and can be detected at virtually any stage of plant development.

There are 5 conditions that characterize a suitable molecular marker

1) Must be polymorphic

2) Co-dominant inheritance

3) Randomly and frequently distributed throughout the genome

4) Easy and cheap to detect

5) Reproducible

Molecular markers can be used for several different applications including: germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization and phylogenic analysis (Singh, 2008).

2.7.2. Different types of molecular markers

1) Randomly amplified polymorphic DNA Markers (RAPD)

RAPD was the first PCR based molecular marker technique developed and is far the simplest. Short PCR primers (approximately 10-12 bases) are randomly selected to amplify random DNA segments throughout the genome. The resulting amplification product is generated at the region flanking a part of the 10 -12 bp priming sites in the orientation. RAPD often shows a dominant relationship as the primer is unable to distinguish between homozyogotes and heterozygotes. RAPD bands of different strains or species can be compared. They can be used to construct RAPD maps (Gupta *et al.*, 2010). RAPD obviates the need to work with radioisotopes and is faster in obtaining results (Iqbal *et al.*, 2010). RAPD products are usually visualized on agarose gels stained with ethidium bromide.

2) Restriction Fragment Length Polymorphism (RFLP)

RFLP is generated by the presence or absence of a recognition site for the same restriction endonuclease in the same region of a chromosome from the different individuals of a species. As a result the concerned restriction enzyme produces fragments of different length representing the same chromosome region of different individuals. These differences are detected by gel electrophoresis

combined with hybridization with a labelled probe specific for that chromosome region.

3) Simple Sequence Repeats (SSR)/Microsatellites

Simple sequence repeats are present in the genomes of all eukaryotes and consists of several to over a hundred repeats of a 1-4 nucleotide motif. The sequences flanking these microsatellites are often conserved and can be used to design primers. These primers can be designed by constructing a novel genomic library and sequencing segments of the subject genome. Already discovered sequences (ie. GENEBANK online database) can also be searched for SSRs and primers cen be designed from that. Polymorphism is based on the number of tandem repeats and therefore the length of the PCR products. SSR is a co dominant marker like RFLP and is usually visualized on metaphor agarose or polyacrylamide gels (Singh, 2008).

4) Amplified Fragment Length Polymorphism (AFLP)

AFLP is the latest form of marker assisted selection and is a highly sensitive method based on the combined concepts of RFLP and RAPD. This technique is applicable to all species and gives very reproducible results. The basis of AFLP is the PCR amplification of restriction enzyme fragments of genomic DNA. Typical results give 50-100 bands despite selective nucleotides and rare/frequent selection. This high number of bands eases analysis by providing more chance of polymorphism (Singh, 2008).

5) Sequence Tagged sites (STSs)

A sequence tagged site is any site on the genome that is unambiguously defined in terms of flanking primers that are used for PCR amplification of this site. It is a short unique sequence (200-500) that can be amplified by PCR, which

identifies a known location on a chromosome (Olson *et al.*, 1989). Examples of STSs are Sequence tagged microsatellite (STMs), Sequence characterized amplified regions (SCARs), Cleaved amplified polymorphic sequence (CAPs). STS-based PCR produces a simple and reproducible pattern on agarose or polyacrylamide gel. In most cases STS markers are co-dominant.

6) Sequence Characterised Amplified Region (SCAR's)

A SCAR is a genomic DNA fragment at a single genetically cloned locus that is identified by PCR amplification using a pair of specific oligonuclotide primers. It is similar to the RAPD method but uses longer primers, which are generally composed of between 18 and 24 bases. By increasing the specificity of the primers, the results become more reproducible and more specific (Hernandez *et al.*, 1999).

2.8. APPLICATIONS OF RAPD

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

Lim *et al.* (1999) did RAPD analysis of some species of the genus Vanda (Orchidaceae) and the related genus *Ascocentim.* Results indicated that strap leaved Vanda species including *Vanda sanderina* and *Ascocentrim miniatum* were more closey related to each other than the terete leaved Vanda species.

Hernandez *et al.* (1999) reported development of SCAR's by direct sequencing of RAPD products for marker assisted selection in wheat. RAPD

markers generated by mixtures of two different primers were developed for *Hordeum chilense* x *Triticum aestivum* and its parents. Ten RAPD bands were selected and eight of them were converted to dominant SCAR markers.

Bi-hao *et al.* (2009) reported inheritance and identification of SCAR marker linked to bacterial wilt resistance in brinjal. The combination of F_1 obtained by a cross between a highly resistant and susceptible bacterial wilt eggplants and its F_2, BC1 were inoculated with *Ralstonia* race 1. A 762 bp molecular marker linked to bacterial wilt resistance gene of eggplant was identified by BSA and SCAR marker linked to bacterial wilt resistance gene was obtained.

Nematzadh *et al.* (2004) mapped the gene for aroma in rice (Oryza sativa L) by bulk segregant analysis using RAPD marker. DNA samples of homozygous aromatic and homozygous non aromatic plants identified on the basis of progeny tests were bulked and used. Primers AG 8 and AN 1 produced polymorphism.

Archak *et al.* (2002) analysed the genetic diversity of 27 tomato cultivars grown in India with RAPD markers generated with 42 random primers and reported low levels of polymorphism in tomato cultivars by RAPD. They reported difficulties for molecular characterization of cultivars of diploid autogamous solanaceous species.

Clain *et al.* (2004) reported homogeneity between accessions of *Solanum torvum* regarding high bacterial wilt tolerance. It was based on the genetic homogeneity (zero percent polymorphisms) revealed using 168 RAPD primers.

Singh *et al.* (2005) used RAPD technique as a tool for assessing genetic diversity and species relationships among 28 accessions of eggplant representing 5 species. The study showed that *S. incanum* is closest to *S. melongena* followed by *S. nigrum*.

Kizhakkayil *et al.* (2008) did molecular profiling using RAPD and clustering of traditional black pepper samples from India, Indonesia, Vietnam and Malaysia using 23 random decamer primers. They revealed a comparatively high genetic similarity within the samples from a particular country than between any two countries. The UPGMA dendrogram constructed based on similarity coefficient revealed total of four groups in two different clusters.

Nouhi *et al.* (2008) tagged a resistance gene to rhizomania disease in sugar beet (*Beta vulgaris*). They used 300 RAPD primers in the analysis of two parents and bulk F_2 population. Genes were tagged using two RAPD primers and one of the markers was OP-09_{1150} which is 25 cM apart from R_{Z1} gene in coupling phase. The second marker was OP-AN 9_{600} which was 13.7 cM apart from R_{Z1} gene and in repulsion phase.

Zhang *et al.* (2008) developed SCAR markers linked to the gene inducing beta-carotene accumulation in Chinese cabbage based on RAPD and AFLP ,by performing a bulked segregant analysis (BSA) using a doubled haploid (DH) population derived from the F_1 cross between 91-112 (white head leaves) and T12-19 (orange head leaves) via microspore culture. Two RAPD markers—OPB01- 845 and OPAX18-656—and 1 AFLP marker, namely, P67M54-172, were identified to be linked to the *or* gene, and they were successfully converted into the SCAR markers SCR-845, SCOR204, and SCOR127, respectively.

Kumari (2008) did molecular characterization of chilli genotypes for tagging bacterial wilt resistance gene using RAPD marker. Resistant and susceptible genotypes along with bulked populations were used for analysis. OPS 1 primer amplified a DNA fragment of 1.24 kb in resistant parent and resistant bulk and SCAR primers were designed. But SCAR primers couldn't distinguish resistant and susceptible genotypes. Sudhamayee (2010) reported sex determination in nutmeg using RAPD marker. Sixty seven primers were screened and among them one primer OPK 01 amplified reproducible female specific band

(1.1 kb) in bulked female and individual female samples and SCAR primers were designed.

2.9. RAPD FOR TAGGING RECESSIVE GENES

Zhang *et al.* (1996) mapped bacterial blight resistance gene *Xa-13* in rice using RAPD and RFLP markers. The recessive gene *Xa-13* confers resistance to Philippines race 6 of *Xanthomonas oryzae*. From 260 random decamer primers one primer OPAC 05 amplified specific band of 0.8 kb from DNA of resistant plants.

Johnson *et al.* (1997) reported molecular tagging of the recessive *bc-3* gene which confers resistance to bean common mosaic virus using RAPD technique in Andean common bean. Bulked segregant analysis was employed to identify RAPD markers linked to the *bc-3* locus. The ROC11/350/420 marker was codominant with the *bc-3* gene and the ROC20/460 marker was dominant and linked in trans.

Rao (2003) tagged recessive bacterial blight resistant gene in rice using RAPD and SSR markers. They used 80 RAPD primers and one primer OPA 12 amplified one polymorphic band between resistant and susceptible parent.OPA 12 marker was linked to resistance gene in Ajaya variety at a distance of 5.0cM.

Sajid-Ur-Rahman *et al.* (2008) identified a DNA marker for tagging nectariless trait in cotton using random RAPD technique. Double recessive distance of 10 cM from nectariless loci genotype produces nectariless phenotype which confers resistance to pink boll worm. More than 300 RAPD primers were used. Of these the primer GLE 11 was linked to nectariless trait. The primer amplified a polymorphic DNA fragment of 1145 bp between parents and bulk. Fifteen DNA samples of only homozygous F_2 plants (nectaried and nectariless)

were used to construct bulk. The marker was present at a distance of 10 cM from nectariless trait loci.

Singh *et al.* (2005) developed a RAPD-based SCAR marker SCA 12 linked to recessive gene conferring resistance to anthracnose in sorghum [*Sorghum bicolor* L.) Moench. On bulked segregant analysis, primer OPA 12 amplified a unique band of 383 bp only in the resistant parent G 73 and resistant bulk. Based on the sequence of cloned RAPD product, a pair of SCAR markers SCA 12-1 and SCA 12-2 was designed using the Mac Vector program, which specifically amplified this RAPD fragment in resistant parent G 73, resistant bulk and respective RILs.

2.10. APPLICATIONS OF OTHER MOLECULAR MARKERS

Fukuoka *et al.* (1994) mapped STS in rice by single strand conformation polymorphism (SSCP). Seventy primer pairs were designed from the sequence data available to amplify DNA regions as STSs, and 39 of these STSs were found to generate SSCP between *japonica* rice (Nipponbare) and *indica* rice (Kasalath) in at least one of the experimental conditions. The maps of DNA fragments amplified from 186 F_2-plant DNAs with 17 primer pairs were successfully determined.

Collins *et al.* (2001) developed polymerase chain reaction-based, STS markers for fine mapping of the barley (*Hordeum vulgare*) *Ror1* gene required for broad-spectrum resistance to powdery mildew (*Blumeria graminis* f. sp. *hordei*). After locating *Ror1* to the centromeric region of barley chromosome 1H using a combined AFLP/RFLP)approach, sequences of RFLP probes from this chromosome region of barley and corresponding genome regions from the related grass species oat (*Avena* spp.), wheat, and *Triticum monococcum* were used to develop STS markers.

Sharopova *et al.* (2002) developed 1051 novel SSR markers for maize from microsatellite-enriched libraries and by identification of microsatellite-containing sequences in public and private databases. Three mapping populations were used to derive map positions for 978 of these markers. The main mapping population was the intermated B73 x Mo17 (IBM) population. By mapping this intermated recombinant inbred line population development of a new high-resolution map resource for maize was developed.

Moreno-Vázquez *et al.* (2003) developed SNP -based codominant markers for tagging *cor* gene, a recessive gene conferring resistance to corky root rot (*Rhizomonas suberifaciens*) in lettuce (*Lactuca sativa*). Menezes *et al.* (2004) reported 21 AFLP primers producing bands specific to genotypes resistant to tomato spotted wilt virus and 5 primers producing bands specific to susceptible genotypes from 170 AFLP primers studied.

Murayama *et al.* (2003) identified DNA markers linked to a fertility restorer (*Rf*) gene for Ogura cytoplasmic male sterility in radish (*Raphanus sativus* L.). AFLP analysis was performed on bulked DNA samples from male-sterile and male-fertile radishes. Using 32 AFLP primer pairs, one AFLP fragment (AFLP190) which is specific to the bulked DNA samples from male-fertile F_2 plants was identified. AFLP190 was characterized by molecular cloning and nucleotide sequencing, and was converted to a sequence-tagged site (STS) marker, STS190. A linkage analysis performed in 126 individuals of two independent F_2 populations showed tight linkage of STS190 to the *Rf* gene. The rate of recombination between the marker and *Rf* was estimated to be less than 1%, making STS190 1.2 cM from the gene.

Hoffman *et al.* (2003) converted an RAPD marker to an STS marker which was helpful in identifying barley variety Stander from Robust which has helped American malting and brewing industries.

Onozaki *et al.* (2004) reported that a RAPD-derived STS marker was linked to a bacterial wilt (*Burkholderia caryophylli*) resistant gene in carnation. They aimed to identify RAPD markers associated with the genes controlling bacterial wilt resistance in a resistant segregating population of 134 progeny plants from a cross between Carnation Nou No.1 (a breeding line resistant to bacterial wilt) and Pretty Favvar (a susceptible cultivar) using 505 primers. Out of 8 RAPD markers identified by bulk segregant analysis, a locus with large effect on bacterial wilt resistance was mapped around WG44-1050 through QTL analysis. Then WG-1050 was converted to a STS marker for marker assisted selection.

Bornet *et al.* (2004) analysed 12 strains of phytoplanktons with ISSR markers. The 6 primers gave 223 polymorphic markers that clearly and easily distinguished all 12 strains (mainly toxic ones) and gave 187 polymorphic markers among the Alexandrium and the Pseudo-nitzschia species. ISSR amplifications also indicated a large occurrence of simple sequence repeat (SSR) in phytoplankton genomes, especially in Pseudo-nitzschia, and showed their usefulness to cluster intra and inter species.

Furini and Wunder (2004) reported molecular analysis of 94 Solanum accessions, including eggplants and related species using AFLP technique. Moon (2006) identified AFLP markers linked to tomato spotted wilt virus (TSWV) resistance gene in tobacco. A population of 88 F_2 plants and 23 doubled haploid lines were screened with 32 markers and a 2.5 cM map with 24 markers was constructed. Eleven AFLP fragments between 100 to 400 bp in size linked in coupling phase to TSWV resistance were isolated and sequenced to develop PCR based markers. Four AFLP fragments were successfully converted to sequence characterized amplified region (SCAR) markers.

Fayyaz *et al.* (2007) used resistance gene analogue (RGA) and AFLP markers in double haploid population of wheat derived from a cross between two

parents O 734 and F 1054 to construct a preliminary genetic map. Mishra *et al.* (2009) did AFLP analysis of 29 tea clones which revealed a high degree of polymorphism in genomes of different clones.

Divakaran, A (2007) reported molecular characterization of tomato with special reference to tomato leaf curl virus (ToLCV) resistance using RAPD and AFLP markers. Trait-related markers were detected in a single primer pair in AFLP assay, while RAPD didn't give any clear demarcation with respect to ToLCV resistance/susceptibility. Karumannil (2007) also reported that AFLP technique was better to display the genetic diversity among tomato genotypes compared to RAPD.

Wen *et al.* (2008) developed an STS marker tightly linked to the *Yr2b* gene conferring resistance against wheat stripe rust using resistance gene-analog polymorphism technique (RGAP) in wheat.

2.11. BULKED SEGREGANT ANALYSIS

BSA is a method to identify molecular markers linked to gene of interest without having to construct a map of the genome (Michelmore *et al.*, 1991). Instead of screening dozens of individuals to test for linkage, DNA bulks with fewer than 10 individuals are normally bulked with random primers saving time and resources. Another advantage of BSA is that only polymorphic bands potentially linked to the gene of interests are scored. Two bulked DNA samples are generated from a segregating population from a single cross. Each pool, or bulk, contains individuals that are identical for a particular trait or genomic region but arbitrary at all unlinked regions. The bulks are screened for differences using restriction fragment length polymorphism probes or random amplified polymorphic DNA primers. Bulked segregant analysis has several advantages over the use of near-isogenic lines to identify markers in specific regions of the genome.

Michelmore *et al.* (1991) used bulked segregant analysis to identify three random amplified polymorphic DNA markers in lettuce linked to a gene for resistance to downy mildew. The number of individuals in the bulk was 14.They showed that markers can be reliably identified in a 25 centimorgan window on either side of the targeted locus.

Rifty *et al.* (1998) mapped the *Rk* gene in tobacco which confers resistance to race 1 and 3 of root-knot nematode *Meloidogyne incognita* using RAPD marker. Resistant parent, susceptible parent, and bulk population were screened with 1500 random decamer primers that amplified bands polymorphic between Rk-R nd Rk-S lines.Three root-knot nematode resistant bulks and three root-knot-nematode bulks were created by pooling the DNA of 8-10 lines.

Leaf rust caused by *Mefumpsora medusae* is a major disease problem *on Populus deltoides.* Tabor *et al.* (2000) identified molecular markers linked to a *M. medusae* resistance locus (Lrd l) that was segregating 1:l within an intraspecific *P. deltoides* family (C9425DD). Using bulked segregant analysis they identified two random amplified polymorphic DNA (RAPD) markers ($OPGl0_{340}$ and $OPZ18_{900}$ts) that are linked to Lrd 1.

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

Ouedraego *et al.* (2002) reported AFLP analysis in combination with bulked segregant analysis of F_2 mapping population to identify molecular markers linked to cowpea genes conferring resistance to *Striga geseroioides* race I. Seven AFLP markers linked to striga resistance genes were identified.

Gangashetti *et al.* (2004) reported inheritance of elongated uppermost internode and identification of RAPD marker linked to recessive *eui* gene in rice. In F_{2-3} and test cross EUI exhibited a monogenic recessive inheritance.BSA using RAPD marker identified an association of marker OPAG 01 1000 with EUI which was further confirmed by co-segregation and linkage analysis.DNA from 10 each of EUI and non-EUI F_2 plants in equal quantity formed the EUI and non-EUI bulks respectively.

Singh *et al.* (2006) used a total 29 resistant and 20 susceptible recombinant inbred lines (RILs) derived from a HC 136 x G 73 cross was used for bulked segregant analysis to identify a RAPD marker closely linked to a gene for resistance to anthracnose in sorghum. On BSA a primer OPA 12 amplified a unique band of 383 bp only in resistant parent and resistant bulk.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Development of a molecular marker for bacterial wilt resistance in brinjal (*Solanum melongena* L.) varieties Surya and Swetha" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2009-2011. The materials used and methodologies adopted are discussed in this chapter.

3.1. MATERIALS

3.1.1. Plant Materials

The genotypes selected for the study are Surya, Swetha and Pusa Purple Long (Plate 1). Among them Surya and Swetha are resistant to bacterial wilt caused by *R. solanacearum* and Pusa Purple Long is susceptible. Surya has non-prickly stem and leaves. Leaves are green with violet tinged vein. Fruits are glossy, oval, purple and medium sized. Pusa Purple Long is a dwarf statured variety with elongated and purple coloured fruits. Swetha has bushy and semi-erect growth with white and medium long fruits. The seeds of Surya and Swetha were collected from Department of Olericulture, College of Horticulture and Pusa Purple Long were collected from IARI, New Delhi.

The details regarding the source of resistance have been discussed in introduction. F_1 progenies of the cross Surya x Pusa Purple Long were raised by controlled crossing. Single plant F_2 progenies were raised by selfing F_1 plant and were screened for bacterial wilt incidence. The resistant and susceptible segregants from single plant were used for genotypic analysis by RAPD markers.

3.1.2. Laboratory chemicals and glassware

The chemicals used in the study were of good quality (AR grade) procured from Merck India Ltd., SRL, HIMEDIA and SISCO Research Laboratories. The Taq DNA polymerase, dNTP's, Taq buffer and molecular weight marker (lambda

a.Resistant parent Surya

b.Resistant variety Swetha

c. Susceptible parent Pusa Purple Long

Plate 1.Genotypes selected for the study

DNA/Hind 111±EcoR1 double digest) were supplied by Bangalore Genei. RNase A from Sigma, USA was used. The random primers were obtained from Operon technologies, USA and were synthesized from Sigma Aldrich Chemicals, Bangalore. The plastic wares used for the study were purchased from Tarsons India Ltd. and Axygen, USA.

3.1.3 Equipment and machinery

The equipments available at the Centre for Plant Biotechnology and Molecular Biology and the Bioinformatics Centre, College of Horticulture were used for the present study. Centrifugation, in DNA isolation was done at KUBOTA centrifuge. The PCR was done in Eppendorf Master Cycler (Eppendorf, USA) and agarose gel electrophoresis was done in horizontal gel electrophoresis system (BIO-RAD). The Herolab ® UV transilluminator was used for checking DNA after electrophoresis. BIO-RAD Gel DOC imaging system was used for imaging the gel. The list of laboratory equipments used for the study is provided in Appendix 1.

3.2. METHODS

3.2.1. Development of segregating F_2 population

3.2.1.1. Development of F_1 plants

The bacterial wilt resistant variety Surya and susceptible variety Pusa Purple Long were grown in pots containing sterilized soils as per the package of practices of Kerala Agricultural University. The variety Surya was crossed with Pusa Purple Long to develop the F_1 progenies. Flowers of Surya were emasculated at 5.30 pm prior to the day of anthesis and were covered with butter paper. On the day of anthesis, pollen from Pusa Purple Long were dusted on emasculated flowers of Surya by 6.30 am and covered with butter paper. The pollinated flowers were checked for fruit set, four days after pollination.

The mother plant was sprayed with carbaryl 0.15 per cent at an interval of 15 days to prevent fruit and shoot borer attack. Seeds from ripe F_1 fruits were collected and were sown for raising F_1 plants.

3.2.1.2. Development of F_2 progenies

Flowers of F_1 plants were covered with butter paper prior to the day of anthesis to allow self pollination alone. The selfed F_1 plants produced F_2 generation seeds. The F_2 plants raised from single F_1 plant were used as mapping population.

3.2.2. Standardization of artificial inoculation technique

Two methods of inoculum delivery system ie. soil drenching with wounding and stem puncture method were compared. The seedlings of susceptible variety Pusa Purple Long, resistant variety Surya and F_1 plants were used for the study. One month old seedlings were used for screening.

The seeds of selected genotypes were initially made to germinate in pots containing sterilized medium of sand, soil and farmyard manure in the ratio of 1:1:1. Media was sterilized with 40 percent formaldehyde solution (1:30). This solution was applied at the rate of 1L per pot and covered with polythene sheet. After one week the polythene sheet was removed and the pots were kept open for another seven days to remove the residual formalin content. The seeds were sown for germination.

Thirty days old individual seedlings at 3-4 leaf stage were transplanted to polybags containing sterile soil medium. Fresh bacterial ooze collected from the wilted plants was used for inoculation. The optical density (OD) of bacterial ooze collected in 100ml sterile distilled water was adjusted to 0.3 at 600nm containing 10^8 cfu/ml. The bacterial ooze with this population count was used for inoculation.

In each treatment 20 F_1 plants and 10 each of Surya and Pusa Purple Long were screened. Five plants were maintained as control without inoculation. The details of two methods tried are given below:

1. Soil drenching with wounding

The lateral roots of each transplanted seedlings were cut along one side of the plant by inserting the blade of the knife 1.5 cm away from the collar region to a depth of about 4 cm. Then it was transplanted in polybags containing sterilized soil and the soil was drenched by pouring 30 ml of the inoculums around the base of the seedlings.

2. Stem puncture method

A drop of bacterial suspension was placed in the axils of second and third expanded leaves below the stem apex. A needle was forced into the stem through the drop. A piece of cotton dipped in bacterial suspension was then placed in the punctured axils to ensure sufficient inoculums. The treatment was given one week after transplanting.

Days taken for initiation of wilting and percentage of plants wilted after one month were observed. Treatments were compared for these two characters. The association of pathogen with wilt was confirmed with ooze test.

Based on the observations made on disease reaction, the per cent wilt incidence was calculated using the following formula:

$$\text{Per cent of wilt incidence} = \frac{\text{No. of plants affected by wilt}}{\text{Total no. of plants under observation}} \times 100$$

3.2.2.1. Phenotyping of genotypes for bacterial wilt resistance

The F_1 and F_2 progenies of the cross Surya x Pusa Purple Long along with their parents were artificially inoculated with *R. solanacearum* using stem puncture method. Thirty five F_2 plants and ten plants each of Surya and Pusa Purple Long were inoculated. Plants were observed for one month. The severity of wilt incidence was scored according to the resistance/susceptibility based on the scoring system proposed by Mew and Ho (1976) as shown below:-

Disease Rating	**Per cent survival**
R (Resistant)	80 or above
MR (Moderately resistant)	60-80
MS (Moderately susceptible)	40-60
S (Susceptible)	less than 40

From the screened F_2 population 5 resistant and 5 susceptible plants were selected for bulk segregant analysis.

3.2.3. MOLECULAR CHARACTERISATION OF BRINJAL GENOTYPES WITH RAPD MARKER

Molecular characterization of selected brinjal genotypes and F_2 progenies (Plate 2) for tagging bacterial wilt resistance gene was done with RAPD.

3.2.3.1. Standardisation of genomic DNA isolation technique

Isolation of good quality genomic DNA is an important parameter for RAPD analysis. The procedures reported by Doyle and Doyle (1987) and Rogers and Bendich (1994) with slight modifications were tested for DNA isolation and the quality of DNA obtained was compared. The quality of DNA isolated was

compared by gel electrophoresis and nanodrop method. DNA was isolated from tender leaves collected early in the morning.

3.2.3.1.1. Procedure by Doyle and Doyle (1987)

Reagents

1. Extraction buffer (4X)
2. Lysis buffer
3. TE buffer
4. Isopropanol
5. Chloroform: isoamyl alcohol(24:1,v/v)
6. 5% Sarcosin
7. Ethanol 100 and 70%

Details composition of reagents of are provided in the Appendix II

Procedure

1g leaf sample was ground to a fine powder in excess of liquid nitrogen using a prechilled mortar and pestle. A pinch of sodium metabisulphate and 50µl of β-mercaptoethanol were added. Later 3 ml extraction buffer was also added. The homogenate was transferred to a 50 ml oakridge tube containing 4 ml prewarmed lysis buffer and 1ml sarcosin. The contents were mixed well and maintained at 65^0C for 15mts. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged at 10,000 rpm for 15 mts at 4^0C. The contents got separated into three distinct phases. The upper aqueous phase containing DNA was pipetted out into a fresh 50 ml oakridge tube. Then 0.6 volume of ice cold chilled isopropanol was added and the contents were mixed gently. The sample was then incubated at -20 ^{0}C for one hour to precipitate the DNA completely. The DNA was then pelleted by centrifuging at 10000 rpm for 15 minutes at 4^0C. The isopropanol was poured out and the pellet was washed with 70 percent alcohol. The pellet was air dried to remove alcohol and finally dissolved in 100 µl TE buffer.

3.2.3.1.2. Procedure by Rogers and Benedich (1994) with modification

In the modified procedure a pinch of sodium meta bisulphate, 50 µl β-mercaptoethanol were added to reduce polyphenol interference.

Reagents

1) 2X CTAB buffer

2)10% CTAB solution

3) Chloroform: Isoamyl alcohol l(24:1)

4) Isopropanol

5)100% ethanol

6) 70% ethanol

The details of preparation of reagents are provided in Appendix II

Procedure

1 g leaf sample was taken. It was ground in a prechilled mortar and pestle in presence of a pinch of sodium meta bisulphate and 50 µl β-mercaptoethanol in liquid nitrogen. This was transferred to a 50 ml fresh oakridge tube containing 7 ml prewarmed 2X CTAB extraction buffer. The mixture was incubated at 65°C for 20-30 minutes with occasional mixing by gentle inversion. Then equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion. Then it was centrifuged at 10,000rpm for 15 mts at 4°C. Aqueous phase containing DNA was removed by a wide-bore pipette and transferred to a clean oakridge tube and $1/10^{th}$ the volume of 10 % CTAB was added. Then again equal volume of Chloroform: isoamyl alcohol was added and mixed by inversion to emulsify the contents. Then it was centrifuged at 10,000rpm for 15 mts at at 4°C. Aqueous phase was removed by a wide-bore pipette and transferred to a clean tube and add 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till the DNA precipitates. It was kept at -20^0C for half an hour. Then centrifuged at 10,000 rpm for 15 mts. The supernatant was gently poured off and drained well. The pellet was washed in 70% ethanol followed by 100% ethanol. Then it was

centrifuged for 3 mts at 10,000rpm and ethanol was decanted. The pellet was air dried and dissolved in 200 µl TE buffer and stored at -20^{0}C.

3.2.3.2. Purification of DNA

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

Reagents

1) Phenol: chloroform mixture (1:1, v/v)
2) Chilled isopropanol
3) 70 per cent ethanol
4) TE buffer
5) Chloroform: Isoamyl alcohol (24:1, v/v)
6) One per cent RNase

One per cent solution of RNase A was prepared by dissolving the same in TE buffer at 100^{0}C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20^{0}C.

Proccdure

RNase solution (2 µl) was added to 100 µl DNA sample and incubated at 37^{0}C in dry bath (Genei, Thermocon) for 1 hour. The volume was made up to 250 µl with distilled water and equal volume of Phenol: Chloroform mixture was added. This was then centrifuged at 12,000X g for 10 minutes at 4^{0}C. The aqueous phase was collected in a fresh micro centrifuge tube and added equal volume of chloroform: isoamyl alcohol (24: 1). Again they were centrifuged at 12,000Xg for 10 minutes at 4^{0}C. The above two steps were repeated and finally the DNA was precepitated from the aqueous phase with 0.6 volume of chilled isopropanol. The mixture was then incubated at -20^{0}C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4^{0}C. The pellet of DNA was washed with 70 per cent ethanol. The pellet was air dried and dissolved in 250 µl TE buffer.

3.2.3.3.Assessing the quality of DNA by electrophoresis

The quality assessment of isolated DNA was done by Agarose Gel Electrophoresis (Sambrook *et al.*, 1989).

Reagents and Equipments

1. Agarose – 0.8 per cent (for genomic DNA)
   – 1.4 per cent (for PCR samples)
2. 50X TAE buffer (P^H 8.0)
3. Tracking dye (6X)
4. Ethidium bromide (stock 10 mg/ml; working concentration; 0.5 µg/ml)
5. Electrophoresis unit, power pack, gel casting tray, comb
6. UV transilluminator
7. Gel documentation and analysis system

Composition of reagents is provided in Appendix III.

Procedure

1X TAE buffer was prepared from the 50X TAE stock solutions. Agarose (0.8%) was weighed and dissolved in TAE buffer by boiling. Then ethidium bromide was added at a concentration of 0.5µg/ml and mixed well. The open end of gel casting tray was sealed with a cellotape and kept on a horizontal surface. The comb was placed desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 30 minutes after which the comb was removed carefully. The tray was kept in the electrophoresis unit with the well side directed towards the cathode. 1X TAE buffer was added to the tank. Then DNA sample (5 µl) along with tracking dye (1 µl) was loaded into the wells using a micropipette carefully. λDNA/*Eco*RI+H*ind*III double digest was used as a molecular marker. After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (100V) and current (50 A). The power was turned off when the tracking dye reached 2/3rd length of the gel.

Gel documentation

The gel containing electrophoresed DNA was viewed under UV transilluminator for presence of DNA. The DNA fluoresces under UV light due to ethidium bromide dye. The image was documented in gel documentation system (BIO-RAD imaging system). The gel profile was examined for intactness, clarity of DNA band, presence of RNA and protein.

3.2.3.4 .Assessing the quality and quantity of DNA by NanoDrop method

Nanodrop® ND-1000 spectrophotometer was used for analyzing the purity of DNA. It measures absorbance from 2 ul samples with high accuracy and reproducibility. It estimates the concentration of nucleic acid in the sample based on Beer-Lambert law. The absorbance of nucleic acid samples were measured at a wavelength of 260 nm and 280 nm. The purity of DNA was assessed by OD 260/OD 280. A ratio of 1.8 to 2.0 indicate pure DNA.

The quantity of DNA in the pure sample was calculated using the formula OD 260 = 1 is equivalent to 50 ug double stranded DNA

1OD at 260 nm = 50 ug/ml DNA

Therefore OD 260 X 50 gives the quantity of DNA in ug/ml

3.2.3.5. RAPD (Random Amplified Polymorphic DNA) analysis

The good quality genomic DNA isolated from brinjal leaf samples were subjected to RAPD as per the procedure reported by Williams *et al.*, (1990). Random decamer primers with good resolving power were selected for bulk segregant analysis after an initial screening study of primers for DNA amplification.

3.2.3.5.1. Screening of RAPD primers for bulk segregant analysis (BSA)

Ninety two 10-12 bp primers in the series OPA, OPB, OPC, OPF, OPE, OPU, OPH, OPAH, OPAG, OPL, OPM, RY, RN, RA, SC, RF, AG 8, WG 44, GLE11, RF, R10, R6, and PUC101 were screened for DNA amplification of resistant variety Surya and Susceptible variety Pusa Purple Long as the template. Thirty wilt specific primers as cited by review were also included. Details of the primers used for screening is given in (Table 1 to Table 6). Genomic DNA at the concentration of 50 ng was subjected to PCR amplification using decamer primers. The samples used were the following.

1. DNA of resistant variety Surya
2. DNA of susceptible variety Pusa Purple Long
3. Negative control (without DNA)

The amplification was carried out in an Eppendrof Master Cycler (Eppendrof, USA). The products were gel electrophoresed. The gel profile was examined for resolving of DNA as discrete bands and polymorphism of bands. The band was considered as monomorphic if the primer amplified same molecular weight region in both Surya and Pusa Purple Long and it was considered as polymorphic if present in one of the samples and absent in other. Primers producing an average of five bands were selected for BSA.

Procedure

A master mix without the template DNA was prepared using the reaction mixture for the required number of reactions. From this master mix, 16.5 µl was pipetted out into each PCR tube. Template DNA (2 µl) was added. PCR amplification was performed in a 20 µl reaction mixture as constituted below.

Table 1. List of OPS series decamer primers used in primer screening

Sl.No	Primer	Nucleotide Sequence (5'-3')
1.	OPS 1	GTTTCGCTCC
2.	OPS 2	TGATCCCTGG
3.	OPS 3	CATCCCCCTG
4.	OPS 4	GGACTGGAGT
5.	OPS 5	TCGGCCCTTC
6.	OPS 6	TGCTCTGCCC
7.	OPS 7	GGTGACGCAG
8.	OPS 8	GTCCACACGG
9.	OPS 9	TGGGGGACTC
10.	OPS 10	CTGCTGGGAC
11.	OPS 11	GTAGACCCGT
12.	OPS 12	CCTTGACGCA
13.	OPS 13	TTCCCCCGCT
14.	OPS 14	TCCGCTCTGG
15.	OPS 15	TTCCCCCGCT
16.	OPS 16	TTTGCCCGGA
17.	OPS 17	AGGGAACGAG
18.	OPS 18	CCACAGCAGT
19.	OPS 19	ACCCCCGAAG
20.	OPS 20	GGACCCTTAC
21.	OPS 30	GTGATCGCAG
22.	OPS 34	TCTGTGCTGG
23.	OPS 39	CAAACGTGGG
24.	OPS 80	ACTTCGCCAC
25.	OPS 127	CCGATATCCC

Table 2. List of OPA series decamer primers used in primer screening

Sl. NO	Primer	Nucleotide Sequence (5'-3')
1.	OPA 4	AATCGGGCTG
2.	OPA 8	GTGACGTAGG
3.	OPA 10	GTGATCGCAG
4.	OPA 05	AGGGGTCTTG
5.	OPA 6	GTGATCGCAG
6.	OPA 16	AGCCAGCGAA
7.	OPA 9	ACTGAACGCC
8.	OPA 12	TCGGCGATAG
9.	OPA 22	TGCCGAGCTG
10.	OPA 23	AGTCAGCCAC
11.	OPA 24	AATCGGGCTG
12.	OPA 25	AGGGGTCTTG
13.	OPA 26	GGTCCCTGAC
14.	OPA 29	GGGTAACGCC
15.	OPA 30	GTGATCGCAG
16.	OPA 32	TCGGCGATAG
17.	OPA 34	TCTGTGCTGG
18.	OPA 36	AGTGCATTCA
19.	OPA 38	AGTGCATTCA
20.	OPA 39	AGTGCATTCA

Table 3. List of OPB and OPC decamer primers used in primer screening

Sl No	Primer	Nucleotide sequence (5'-3')
1	OPB 07	GGTGACGCAG
2	OPB 15	GGAGGGTGTT
3	OPC 8	TGGACCGGTG
4	OPC 9	CTCACCGTCC
5	OPC 14	TGCGTGCTTG

Table 4. List of OPE, OPF and OPU decamer primers used in primer screening

Sl No	Primer	Nucleotide sequence (5'-3')
1	OPE	GAGTCTCAGG
2	OPF 5	CCGAATTCCC
3	OPU3	CTATGCCGAC
4	OPU9	CCACATCGGT

Table 5. List of RN and RY decamer primers used in primer screening

Sl No	Primer	Nucleotide sequence (5'-3')
1	RN 11	TCGCCGCAAA
2	RN 12	AAGCCTGCGA
3	RN 19	GGTGAGGTCA
4	RY 01	GTGGCATCTC
5	RY 02	CATCGCCGCA
6	RY 03	ACAGCCTGCT
7	RY 14	GGTCGATCTG
8	RY 17	AGAGCCGTCA

Table 6. List of wilt specific primers used in primer screening

Sl No	Primer	Nucleotide sequence (5'-3')	Referances	Crop
1	OPM 01	GTTGGTGGCT	Bi-hao *et al.*, 2009	Brinjal
2	AG 8	AAGAGCCCTC	Osiru *et al.*, 2001	Tomato
3	WG 44	TCGCGCTTTGGA	Onozaki *et al.*, 2004	Carnation
4	GLE 11	CAATCGCCGT	Yang *et al.*, 2006	Brinjal
5	OPAG 01	CTACGCCTTC	Gangashetti *et al.*, 2004	Rice
6	OPAH 12	GTACTACCTA	Demir *et al.*, 2010	Brinjal
7	OPAH 09	AGAACCGAGG	Doganler *et al.*, 2002	Brinjal

Table 6 continueed

8	OPH 02	TCGGACGTGA	Demir *et al.*, 2010	Brinjal
9	OPL 04	GACTGCACAC	Demir *et al.*, 2010	Brinjal
10	A 02	TGCCGAGCTG	Nedim *et al.*, 2008	Brinjal
11	OPG 16	AGCGTCCTCC	Siri *et al.*, 2009	*Solanum commersonii*
12	OPH 06	ACGCATCGCA	Siri *et al.*, 2009	*Solanum commersonii*
13	RA 12-29	AGGTTGGCTGAT	Yui *et al*,1998	Tomato
14	RF	CCCGTCAGCA	Siri *et al.*, 2009	*Solanum commersonii*
15	SC 10-04	AACCGACACC	Zhu *et al.*, 2005	Brinjal
16	RA 12-84	CCCTCCCCAGCT	Yui *et al.*, 1998	Tomato
17	OPH 12	GTGCCTAACC	Siri *et al.*, 2009	*Solanum commersonii*
18	RA 12-13	TCATCACACCCG	Yui *et al.*, 1998	Tomato
19	OPH 07	CTGCATCGTG	Siri *et al.*, 2009	*Solanum commersonii*
20	RA 10-22	GTCGGGTGAA	Yui *et al.*,1998	Tomato
21	RA 12-74	CACTCGATACGG	Yui *et al.*,1998	Tomato
22	RA 12-41	GACTTGCGCCCA	Yui *et al.*,1998	Tomato
23	R 10	CAGCCGCCCC	Siri *et al.*, 2009	*Solanum commersonii*
24	RA 12-38	TGAGAGCGTACG	Yui *et al.*, 1998	Tomato
25	OPG 03	GAGCCCTCCA	Siri *et al.*, 2009	*Solanum commersonii*
26	SC 10-20	ACTCGTAGCC	Gousset *et al.*, 2005	*Solanum torvum*
27	SC 10-02	GGTCCTCAGG	Mangin *et al.*, 1999	Tomato
28	OPB 17	AGGGAACGAG	Siri *et al.*, 2009	*Solanum commersonii*
29	PUC 101	AGACCGAACA	Tian *et al.*, 2007	Brinjal
30	R 6	GGTGGGGACT	Siri *et al.*, 2009	*Solanum commersonii*

Composition of the reaction mixture for PCR

a) Genomic DNA (50 ng)	- 2.0 µl
b) 10X Taq assay buffer B	- 2 µl
c) MgCl2	- 1.5 µl
d) dNTP mix (10mM each)	- 1µl
e) Taq DNA polymerase (1U)	- 0.3 µl
f) Decamer primer (10 pM)	- 1.5 µl
g) Autoclaved distilled water	- 11.7 µl
Total volume =	- 20.0 µl

The PCR tubes were kept in the thermal cycler and were run using the following programme

1)94^{0}C for 5 minutes-Initial denaturation
2)94^{0}C for 1 minute-Denaturation
3)37^{0}C for 1 minute-Primer annealing
4)72^{0}C for 2 minutes-Primer extension
5) Go to step 2.Repeat 40 cycles
6)72^{0}C for 5 minutes-Final extension
7)4^{0}C for 10 minutes

The amplified products were run on 1.4 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA / E*co*RI+H*ind*III double digest). The profile was visualized under UV (312 nm) transilluminator and documented using BIO-RAD gel documentation system. The documented RAPD profiles were carefully examined for amplification of DNA as bands. Gel profiles screened by ninty two primers were carefully examined for polymorphism and good amplification as bands. Primers exhibiting polymorphism and those having a mean of 5 bands were selected for BSA.

3.2.3.5.2. Bulk Segregant analysis of brinjal genotypes

Bulk segregant analysis was done with resistant parent Surya, susceptible parent Pusa Purple Long, resistant bulk (pooled DNA of 5 resistant F_2 plants), susceptible bulk (pooled DNA of 5 susceptible F_2plants) and Swetha .Twenty two selected primers were used for the analysis. The primers selected for BSA were RY 01, RY 02, RY 03, RN 19, OPS 4, OPS 9, OPS 10, OPS 16, OPS 17, OPA 04, OPA 6, OPA 9, OPA 24, OPA 26, OPA 34, OPA 36, OPL 04, OPF 5, RA 12-41, RF, OPB17, PUC 101. Among them RY 02, OPS 17,OPA 04 were selected for polymorphism of bands. Others were selected as they had produced good amplification of five or more bands.

PCR was carried out as per the procedure followed for screening of primers. The amplified products of five groups of genomic DNA for each primer were run on 1.4 per cent agarose gel using 1 XTAE buffer stained with ethidium bromide with lambda DNA/Hind111+EcoR1 double digest marker. The RAPD profile was documented for 22 decamer primers and was carefully examined for polymorphism among resistant parent, susceptible parent, resistant bulk, susceptible bulk and Swetha. The number of monomorphic and polymorphic bands produced by each primer was recorded and per cent of polymorphism for each primer was calculated as given below:

$$\text{Per cent polymorphism} = \frac{\text{No. of polymorphic bands}}{\text{Total no. of bands}} \times 100$$

Results

4. RESULTS

The study entitled "Development of a molecular marker for bacterial wilt resistance in brinjal (*Solanum melongena* L.) varieties Surya and Swetha was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during 2009-2011. The results of various experiments done are discussed in this chapter.

4.1. DEVELOPMENT OF SEGREGATING GENERATION

Resistant variety Surya was selected as the ovule parent and susceptible variety Pusa Purple Long as the pollen parent. Controlled crosses were made to generate the F_1 generation (Plate 2c). Single F_1 plant was selfed to raise 35 F_2 plants (Plate 2d) and they were used as the segregating population for tagging the bacterial wilt resistance gene. Natural self pollination allowed by bagging the flowers one day prior to anthesis has given F_2 fruit.

4.2. PHENOTYPING OF GENOTYPES FOR BACTERIAL WILT RESISTANCE

4.2.1. Standardisation of artificial inoculation technique

Artificial inoculation technique was standardised in polybag cultured plants of susceptible variety Pusa Purple Long, resistant variety Surya and F_1 generation. Artificial inoculation was done 30 days after sowing with fresh bacterial ooze collected from field, when the seedlings were of 3-4 leaf stage during the month of May. Artificial inoculation was done by 2 methods 1) Stem puncturing 2) Soil drenching with root wounding. Plants were maintained in open condition. The susceptible genotypes showed wilting within 5 days in stem puncture method. In soil drenching with wounding it was eight days. The wilted plants were subjected to ooze test (Plate 3b) and *R. solanacearum* was confirmed to be the causal organism.

a.Resistant parent (Surya)

X

b.Susceptible parent (Pusa Purple Long)

c. F_1Hybrid of Surya and Pusa Purple Long

d. F_2 progenies of Surya X Pusa Purple Long

Plate 2. Development of F_1 and F_2 progenies

Table 7.Standardisation of artificial inoculation techniques using different genotypes

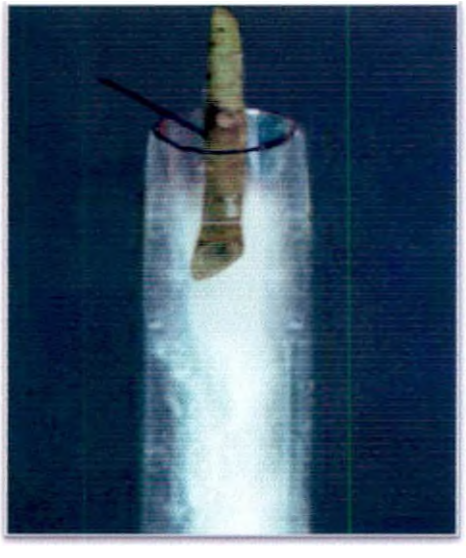
Sl No	Methods	No of plants	Days taken for Initiation of wilting	% of wilting afte one month
1	Stem puncture method	Surya	0	0
		Pusa Purple Long	5	100
		F_1	5	90
2	Soil drenching with wounding	Surya	0	0
		Pusa Purple Long	8	100
		F_1	9	75

Table .8. Phenotyping of genotypes for bacterial wilt incidence

Sl No	Genotype/Generation	No of plants artificially inoculated	Per cent survival	Disease Reaction
1	Surya	10	100	R
2	Pusa Purple Long	10	0	S
3	F_1	20	10	S
4	F_2	35	17	S

Resistant Plant **Susceptible Plant**

a.Phenotyping of genotypes for bacterial wilt incidence



b. Ooze test for confirmation

Plate 3.Symptom of bacterial wilt and its confirmation

In both methods Surya showed 100 per cent resistance and Pusa Purple Long showed 100 per cent susceptibility. In F_1 plants stem puncture method recorded 90 per cent wilting while in soil drenching with root wounding recorded only 75 per cnt wilting after one month (Table 7). So the artificial inoculation technique stem puncture method was selected for screening the F_2 plants for wilt incidence.

4.2.2. Phenotyping of genotypes for bacterial wilt incidence

F_1 and F_2 progenies along with parents Surya and Pusa Purple Long were screened and the results are given in (Table 8). Stem puncture method was used for artificial inoculation. The wilt symptoms occurred with 5 days after inoculation in open condition during the month of October. Symptoms observed were drooping and yellowing of lowermost leaves, followed by complete wilting and death of plants within 10 days. The genotypes were classified as resistant or susceptible according to the classification of Mew and Ho (1976).

F_1 plants showed 90 per cent wilt incidence while F_2 plants showed 83 per cent wilt incidence and both were classified as susceptible (S). Maximum wilt incidence (100%) was observed in Pusa Purple Long and it was categorised as susceptible (S). Minimum wilt incidence was observed in Surya (0%) and it was categorised as resistant (R). The wilted plants showed positive response to bacterial ooze test. From the screened F_2 population five resistant and five susceptible plants were selected for bulk segregant analysis. The DNA of 35 F_2 plants were isolated prior to screening for bacterial wilt reaction.

4.3. MOLECULAR CHARACTERIZATION OF BRINJAL GENOTYPES WITH RAPD MARKER

Molecular characterization of selected brinjal genotypes and segregating F_2 population for tagging bacterial wilt resistance gene was done with RAPD marker (Plate 4).

PUSA PURPLE LONG

SURYA

SWETHA

F_2 PROGENY

Plate 4.Genotypes and generation used for molecular characterization of brinjal genotypes

4.3.1. Standardization of genomic DNA isolation

The protocols reported by Doyle and Doyle (1987) and Rogers and Bendich (1994) were compared for the extraction of genomic DNA from brinjal. The quality of DNA isolated using the aforesaid protocols were assessed using agarose gel electrophoresis. Rogers and Bendich protocol (1994) yielded good quality genomic DNA (Plate 5b). The DNA isolated by this method appeared as clear and distinct band in the agarose gel. The DNA obtained by Doyle and Doyle (1987) method was sheared and contained more RNA contamination (Plate 5a). Hence Rogers and Bendich method (1994) was selected for genomic DNA isolation from brinjal.

4.3.2 .Purification of DNA

The genomic DNA isolated by modified Rogers and Bendich (1994) method showed high degree of RNA contamination. The presence of RNA may interfere with the PCR reactions. So the DNA isolated by modified Roger and Bendich (1994) method was purified by RNase treatment. RNase removed RNA contamination in all samples (Plate 6).

4.3.3. Assessment of quality and quantity of DNA

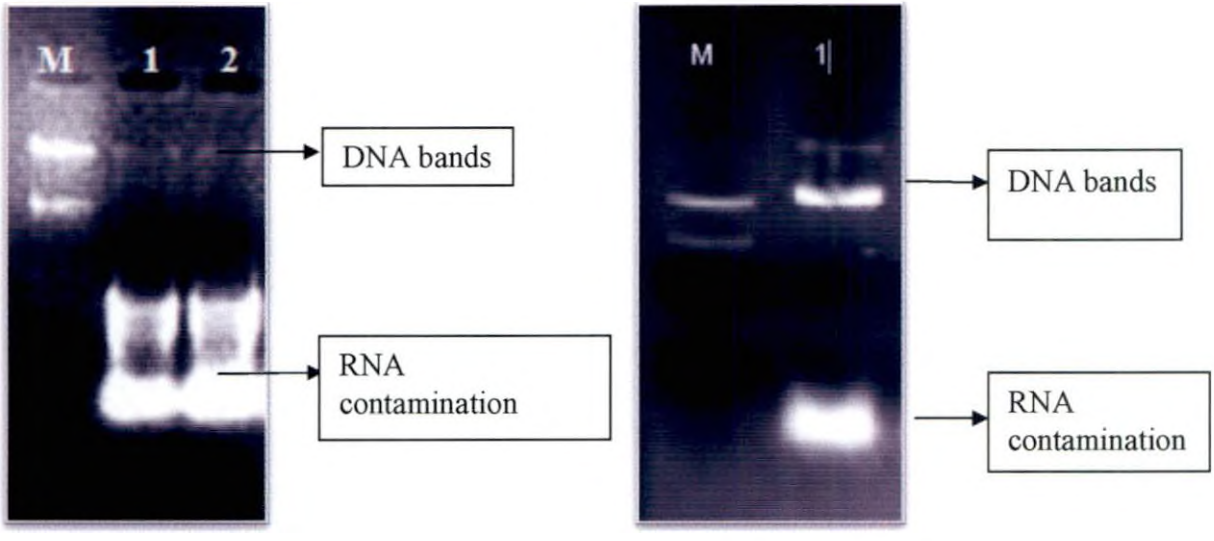
The quantity and quality of DNA isolated by Rogers and Bendich method (1994) was analyzed using Electrophoretic method and NanoDrop® ND-1000 spectrophotometer. In all the DNA samples isolated, intact clear narrow band was observed which indicates non-degraded DNA. The ratio of UV absorbance ($A_{260}/_{280}$) ranged between 1.80 -2.0 which indicated the quality of DNA was good and presented as (Table 9). The DNA thus isolated after appropriate dilutions were used as templates for RAPD analysis.

Table 9.Quality and quantity of DNA isolated from brinjal genotypes as determined by nanodrop method

Genotype/generation	Absorbance			Quantity (ng/µl)	Quality
	A260	A 280	A260/280		
Pusa Purple Long	0.396	0.207	1.91	298	Good
Surya	0.309	0.155	1.99	254	Good
Swetha	0.396	0.207	1.91	282	Good
F_1	0.321	0.171	1.87	306	Good
F2 –S1	0.315	0.166	1.89	233	Good
S2	0.313	0.172	1.82	341	Good
S3	0.310	0.158	1.95	205	Good
S4	0.365	0.193	1.89	232	Good
S5	0.312	0.157	1.99	260	Good
R1	0.378	0.196	1.92	251	Good
R2	0.328	0.176	1.86	248	Good
R3	0.311	0.171	1.83	232	Good
R4	0.343	0.178	1.92	361	Good
R5	0.290	0.161	1.86	240	Good

*S-Susceptible

R-Resistant



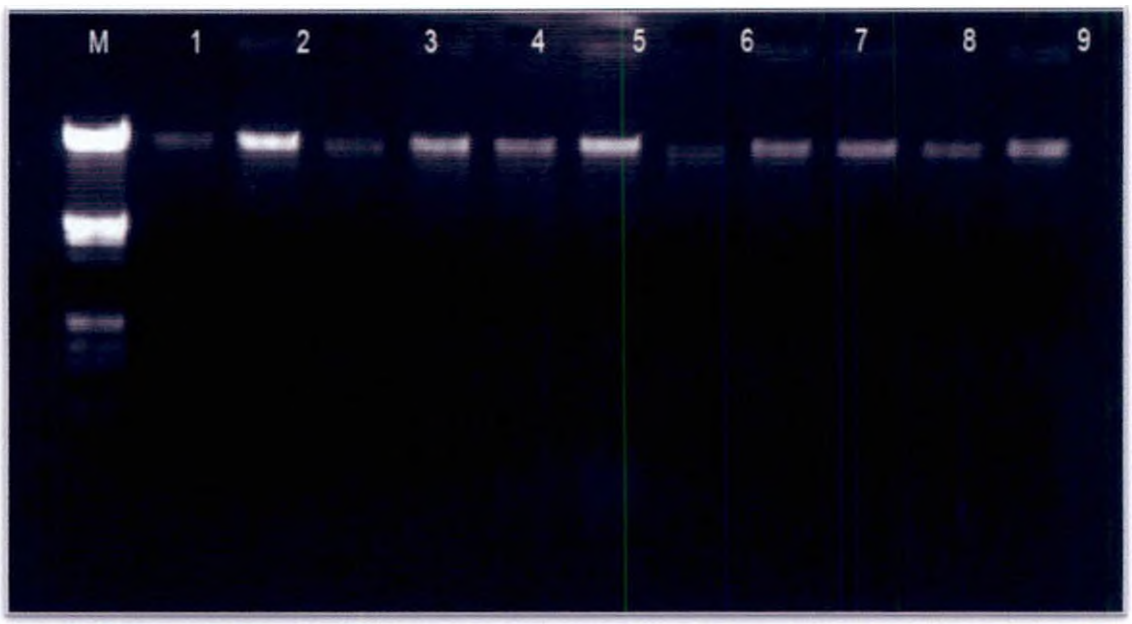
a)Doyle and Doyle method method

b) Rogers and Bendich method

M: λDNA/*Hind* III+ *Eco*RI double digest marker

Lane 1 &2:DNA samples

Plate 5. Standardisation of DNA isolation technique



M: λDNA/*Hind* III+ *Eco*RI double digest marker

Lane 1 -10:DNA samples

Plate 6.DNA samples after RNase treatment

4.3.4. RAPD (Random Amplified Polymorphic DNA) analysis

After isolation of good quality genomic DNA, the brinjal genotypes were subjected to RAPD analysis. The different experiments carried out under this include screening of random primers and bulked segregant analysis.

4.3.4.1. Screening of random primers for RAPD analysis

Ninety two 10-12 bp primers in the series OPA, OPB, OPC, OPF, OPE, OPU, OPH, OPAH, OPAG, OPL, OPM, RY, RN, RA, SC, RF, AG 8, WG 44, GLE11, RF, R10, R6, and PUC101 as shown in (Table 1 to Table 6) were screened for DNA amplification of resistant variety Surya and susceptible variety Pusa Purple Long as the templates. Among them thirty were wilt specific primers. A negative control without DNA was also maintained to check whether any primer –dimer formation is there. The PCR amplification of DNA was done with each primer and products were electrophoresed. The amplification profiles created by gel electrophoresis were examined for number and clarity of bands. The number of bands recorded in resistant and susceptible variety were recorded separately and mean values were found. Primers giving five or more bands were selected for BSA. Gel profiles were also examined for polymorphism of bands between genotypes. Primers giving polymorphism between resistant and susceptible genotypes were also selected for BSA.

4.3.4.1.1. Screening of OPS primers

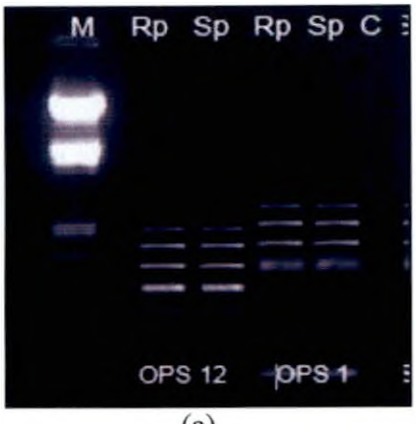
25 primers from OPS series were used for screening. The amplification pattern of OPS series primers are given in (Table 10)

OPS 1

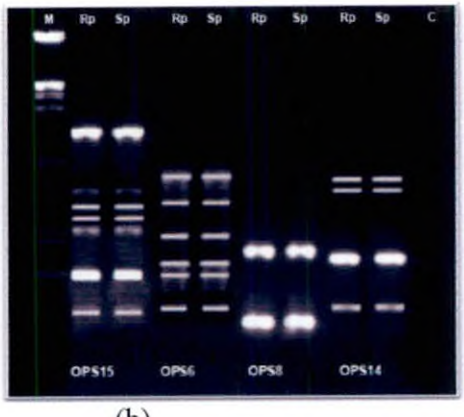
Six amplicons were produced by OPS 1 (Plate 7a). All the bands produced were monomorphic. The band size ranged from 0.5 kb to 3 kb. Four bands produced

Table. 10. Amplification patterns of brinjal genotypes with OPS primers

Sl No	Primer	No of bands		% polymorphism	Amplification pattern
		SP	RP		
1.	OPS 1	6	6	0	Good
2.	OPS 2	5	5	0	Good
3.	OPS 3	8	8	0	Good
4.	OPS 4	10	10	0	Good
5.	OPS 5	4	4	0	Average
6.	OPS 6	6	6	0	Good
7.	OPS 7	4	4	0	Average
8.	OPS 8	2	2	0	Poor
9.	OPS 9	8	8	0	Good
10.	OPS 10	10	10	0	Good
11.	OPS 11	5	5	0	Good
12.	OPS 12	5	4	0	Average
13.	OPS 13	5	1	0	Good
14.	OPS 14	4	4	0	Average
15.	OPS 15	7	7	0	Good
16.	OPS 16	8	8	0	Good
17.	OPS 17	7	5	0	Good
18.	OPS 19	6	6	0	Good
19.	OPS 18	4	4	0	Average
20.	OPS 20	7	7	0	Good
21.	OPS 30	6	6	0	Good
22.	OPS 34	6	6	0	Good
23.	OPS 80	7	7	0	Good
24.	OPS 127	8	8	0	Good
25.	OPS 39	6	6	0	Good



(a)



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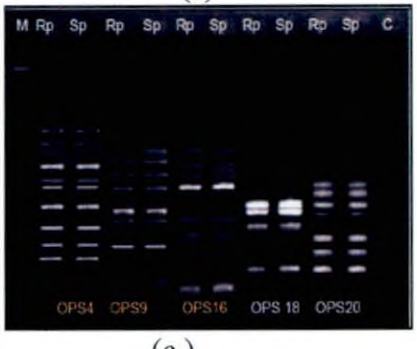

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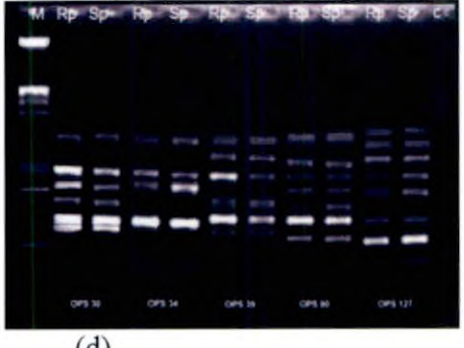

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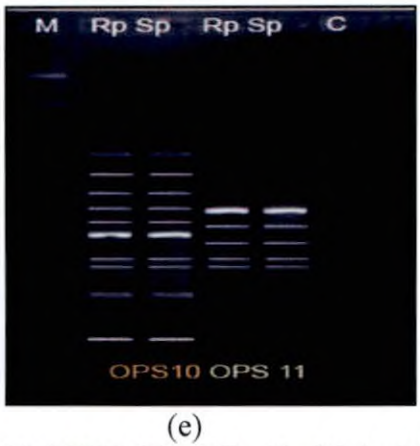

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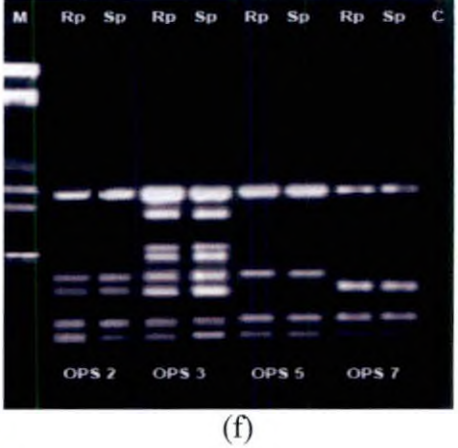


(f)

M: λDNA/*Hind* III+ *Eco*RI double digest marker
RP: Resistant Parent, SP: Susceptible Parent ,C:Negative control
Primers –a)OPS 12,OPS 1 b)OPS 15,OPS 6,OPS 8,OPS 14
c)OPS 4,OPS9,OPS 16,OPS 20 d)OPS30,OPS 34,OPS 39,OPS 80,OPS 127
e)OPS 10,OPS 11 f)OPS 2,OPS3,OPS 5,OPS 7

Plate 7. Screening of brinjal genotypes using OPS series of primers

by this primer were clear and two bands were faint. This primer couldn't distinguish resistant parent from susceptible parent.

OPS 2

OPS 2 produced five monomorphic bands in resistant parent Surya and susceptible parent Pusa Purple Long (Plate 7f). All the bands produced were clear. This primer exhibited zero percent polymorphism. The size of the produced bands ranged from 0.2 kb to 0.9 kb.

OPS 3.

OPS 3 produced a total of 16 amplicons (Plate 7f). It produced 8 monomorphic bands in resistant parent Surya and susceptible parent Pusa Purple Long. All the bands produced by this primer were clear. The band size produced by this primer ranged from 0.2 kb to 0.9 kb. This primer also couldn't distinguish resistant parent from susceptible parent.

OPS 4

OPS 4 produced 10 amplicons (Plate 7c). Seven bands produced by this primer were clear and three bands were faint. Band width ranged from 0.4 kb to 2.1 kb. This primer also produced zero percent polymorphism. This primer was selected for BSA because of its good amplification power.

OPS 5

Four amplicons were produced by OPS 5 (Plate 7f). Size of the bands produced by this primer ranged from 0.4 kb to 1.1 kb. This primer exhibited zero percent polymorphism. All the bands produced by this primer were clear. The size of the amplicons produced by this primer ranged from 0 .4kb-0.kb.

OPS 6

Six amplicons were produced by OPS 6 (Plate 7b). All the bands produced were clear. The size of the amplicons produced by this primer ranged from 0.4 kb to 1.7 kb. This primer also exhibited zero percent polymorphism.

OPS 7

OPS 7 produced four bands in resistant parent Surya and susceptible parent Pusa Purple Long (Plate 7f). All the bands produced by this primer were monomorphic. The size of amplicon produced by this primer varied from 0.1kb to 0.9kb.This primer couldn't distinguish resistant parent from susceptible parent.

OPS 8

OPS 8 produced two amplicons in resistant parent Surya and susceptible parent Pusa Purple Long (Plate 7b). All the bands produced by this primer were monomorphic. The size of the bands produced by this primer ranged from 0.4 kb-0.9 kb. This primer couldn't produce a band unique in resistant variety.

OPS 9

Eight amplicons were produced by OPS 9 (Plate 7c). Seven bands produced by this primer were clear and one band was faint. Size of the band produced by this primer was between 0.3 kb to 2 kb. This primer couldn't produce a unique band in resistant genotypes. This primer was selected for BSA because of its good amplification power.

OPS 10

Ten amplicons were produced by OPS 10 (Plate 7e). .All the bands produced by this primer were monomorphic. It couldn't produce a band unique to resistant parent Surya. The size of the bands produced by this primer ranged from 0.1 kb-2.1 kb. All the bands produced by this primer were clear. This primer was selected for BSA because of its good amplification power.

OPS 11

Five amplicons were produced by OPS 11 (Plate 7e). The size of the amplicons produced by this primer ranged from 0.4 kb to 0.9 kb. The bands produced by this primer were monomorphic.

OPS12

OPS 12 produced five amplicons of size 1.1 kb to 2 kb in resistant genotype and susceptible genotype (Plate 7a). Four amplicons produced were clear and one was faint. It couldn't distinguish resistant genotype from susceptible genotype.

OPS 13

OPS 13 produced five amplicons which were monomorphic. One of the bands produced was clear and all others were faint. The size of the bands produced ranged from 0.5kb to 1.8 kb.

OPS 14

OPS 14 produced four amplicons (Plate 7b). All the four bands were clear and monomorphic. The size of the bands produced ranged from 0.4 kb to 1.7 kb.

OPS15

Seven amplicons were produced by OPS 15 (Plate 7b). Six bands were clear and one band was faint. This primer exhibited zero percent polymorphism. Size of the bands produced by this primer ranged from 0.3 Kb to 2.7 kb.

OPS 16

Eight bands were produced by OPS 16 (Plate 7c). Four bands were clear and four bands were faint. Band size produced by this primer ranged from 0.2kb to 2.1kb.This primer couldn't produce a unique band in resistant genotype. This primer was selected for BSA because of its good amplification power.

OPS17

OPS 17 had produced 7 amplicons (Plate 9f) in resistant parent Surya and 5 amplicons in susceptible parent Pusa Purple Long with a size ranging from 0.3kp to 3.4 kb. All the bands were clear. In the resistant parent two polymorphic amplicons of approximately 1.2 kb and 1.1 kb were present. This primer exhibited 28 % polymorphism. Hence this primer was selected for bulk segregant analysis.

OPS 18

Four amplicons were produced by OPS 18 (Plate 7c). All the four bands were monomorphic. Band size varied from 0.3 kb to 1.3 kb. All the bands were clear.

OPS19

Six amplicons were produced by OPS 19. All the bands were clear. The size of the amplicons produced by this primer ranged between 0.2 kb to 1.9 kb. This primer exhibited zero percent polymorphism.

OPS 20

OPS 20 produced seven amplicons (Plate 7c). All the bands were monomorphic and clear. Band size varied from 0.2 kb to 1.5 kb.

OPS 30

Screening using OPS 30 produced six amplicons (Plate 7d). All the bands were monomorphic and clear. The primer couldn't distinguish resistant parent from susceptible parent. Amplicon size produced by this primer varied from 0.8 kb to 2.1 kb.

OPS 34

Six amplicons were produced by OPS 34 (Plate 7d). All the bands were clear and monomorphic. Size of the amplicons ranged from 0.5 kb to 2.1 kb. This primer exhibited zero percent polymorphism.

OPS 39

Screening using OPS 39 produced six amplicons (Plate 7d). All the bands were monomorphic and clear. The primer couldn't distinguish resistant parent from susceptible parent. Amplicon size produced by this primer varied from 0.8 kb to 2.1 kb.

OPS 80

Seven amplicons were produced by OPS 80 (Plate 7d). All the bands were clear and monomorphic (Plate). Size of the amplicons ranged from 0.8 kb to2.1 kb. This primer exhibited zero percent polymorphism.

OPS 127

Eight amplicons were produced by 127 (Plate 7d). All the bands were clear and monomorphic (Plate). Size of the amplicons ranged from 0.5 kb to 2.1 kb. This primer exhibited zero percent polymorphism.

The primers OPS 4, OPS 9, OPS 10, OPS 16, OPS 17 were taken for bulk segregant analysis.

4.3.4.1.2. Screening of OPA primers

Twenty one primers from OPA series were used for screening. The amplification pattern of OPA series primers are given in (Table 11).

OPA 02

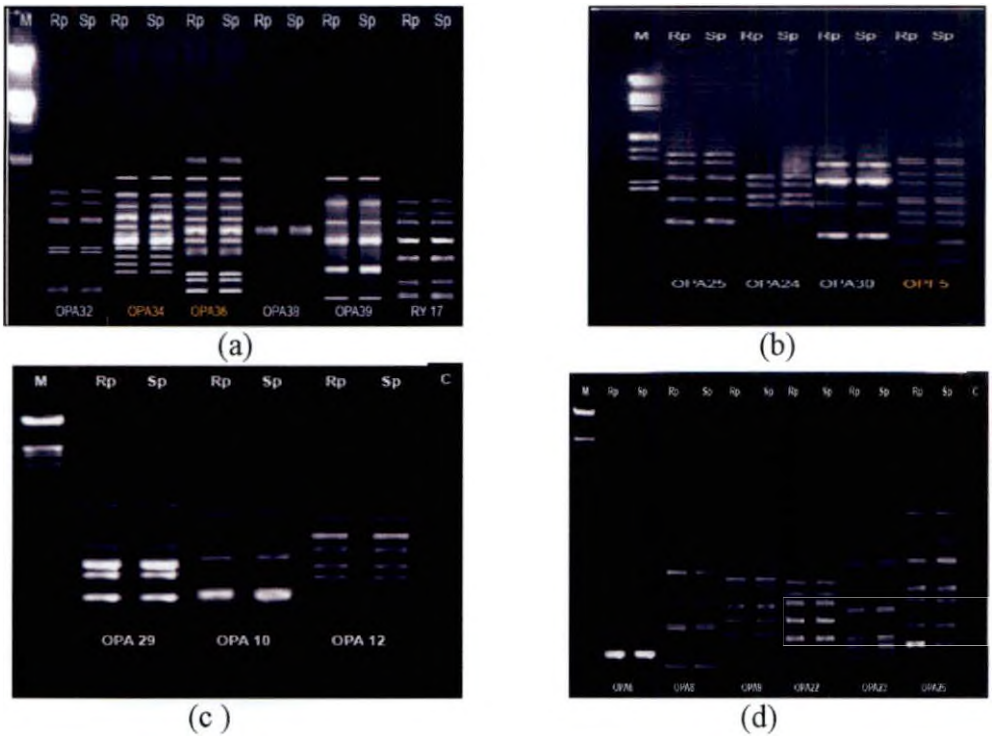
Three bands were produced by this primer (Plate 10b). All the bands were monomorphic.Size of the bands produced by this primer ranged from 0.5 kb to 0.8 kp.

OPA 04

Five bands were produced by this primer (Plate 9f). One band was monomorphic and all other bands were polymorphic. This primer exhibited 80% polymorphism. Size of

Table 11.Amplification patterns of brinjal genotypes with OPA primers

Sl No	Primer	No of bands		% polymorphism	Amplification pattern
		RP	SP		
1	OPA 02	3	3	0	Average
2	OPA 04	1	5	80	Poor
3	OPA 05	5	5	0	Good
4	OPA 6	9	9	0	Good
5	OPA 8	9	9	0	Good
6	OPA 9	7	7	0	Good
7	OPA 10	4	4	0	Average
8	OPA 12	5	5	0	Average
9	OPA 16	5	5	0	Average
10	OPA 22	5	5	0	Good
11	OPA 23	6	6	0	Good
12	OPA 24	4	4	0	Average
13	OPA 25	5	5	0	Good
14	OPA 26	9	9	0	Good
15	OPA 29	6	6	0	Good
16	OPA 30	4	4	0	Average
17	OPA 32	6	6	0	Good
18	OPA 34	11	11	0	Good
19	OPA 36	11	11	0	Good
20	OPA 38	1	1	0	Poor
21	OPA 39	6	6	0	Good



M: λDNA/*Hind* III+ *Eco*RI double digest marker
RP: Resistant Parent
SP: Susceptible Parent, C:Negative control
Primers-a) OPA 32, OPA 34, OPA 36,OPA 38,OPA 39,RY 17
b) OPA 25, OPA 24, OPA 30, OPF 5
c) OPA 29, OPA 10,OPA 12
d) OPA 6,OPA 8,OPA 9,OPA 22,OPA 23,OPA 26

Plate.8. Screening of brinjal genotypes using OPA,OPF and RY series of primers

the amplicons produced by this primer ranged from 0.3 kb to 2.0 kb. This primer was selected for bulk segregant analysis.

OPA 6

Nine bands were produced by OPA 6 (Plate 8d) .All the bands were clear. This primer exhibited zero percent polymorphism. Size of bands produced by this primer ranged from0.3 kb to 1.3 kb. This primer couldn't produce a unique band to distinguish resistant genotypes.

OPA 8

Nine amplicons were produced by OPA 8 (Plate 8d). All the bands were clear. All the bands were present in both genotypes selected for the study. The size of the bands produced by this primer ranged from 0.2kb to 1kb. This primer was selected for BSA because of its good amplification power.

OPA9

Seven bands were produced by OPA 9 (Plate 8d) .All the bands were clear. The size of the bands roduced by this primer ranged from 0.2kb to1kb. This primer couldn't distinguish resistant genotypes from susceptible genotypes. This primer was selected for BSA because of its good amplification power.

OPA 12

Five bands were produced by OPA 12 (Plate 8c).Four bands produced by this primer were clear and one band was faint. All the bands were monomorphic. Size of the amplicons produced by this primer varied from 0.8kb to 1.9kb. This primer couldn't produce a band unique to resistant genotypes.

OPA16

Five bands were produced by OPA 16. One of the bands was clear and all four other bandes were faint. Size of the bands ranged between 0.2 kb to 1.3 kb. This primer couldn't produce a band specific to resistant genotypes.

OPA 22

Five bands were produced by OPA 22 (Plate 8d). Size of the bands produced by this primer varied from 0.3kb to 0.9kb. This primer also exhibited zero percent polymorphism.

OPA 23

Six bands were produced by OPA 23 (Plate 8d). All the bands produced by this primer were clear. This primer exhibited zero percent polymorphism. Size of the bands produced by this primer varied from 0.1 kb to 1.3 kb.

OPA24

Four bands were produced by OPA 24 (Plate 8b). All the bands produced by this primer were clear and monomorphic. Size of the bands produced by this primer ranged between 0.6 kb to 1 kb. This primer was selected for BSA because of its good amplification power.

OPA 25

Five bands were produced by OPA 25 (Plate 8b). All the bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.4 kb to 1.4 kb. This primer couldn't produce a unique band in resistant genotypes.

OPA 26

Nine bands were produced by OPA 26 (Plate 8d).All the bands were clear and monomorphic. Size of the bands produced by this primer ranged from 0.5 kb to 1.9 kb. This primer couldn't distinguish resistant genotypes from susceptible genotypes.

OPA 29

Five bands were produced by OPA 29 (Plate 8c). Three bands were clear and two bands were faint. All the bands produced by this primer were monomorphic. This primer couldn't produce a band specific to resistant genotypes .Size of the amplicons produced by this primer ranged from 0.5kb to 2.1kb.

OPA 30

Four bands were produced by OPA 30 (Plate 8b). Three bands were clear and one band was faint. Size of the amplicons produced by this primer ranged between 0.5 kb to 1.3 kb.

OPA 32

Six bands were produced by OPA 32 (Plate 8a). All the bands were monomorphic and clear. Size of the amplicons produced by this primer ranged between 0.3 kb and 1.3 kb.

OPA34

Eleven bands were produced by OPA 34 (Plate 8a). Nine bands were clear and two bands were faint. Size of the amplicons produced by this primer ranged from 0.5 k b to 2 kb. This primer exhibited zero percent polymorphism. No polymorphism was detected by this primer. This primer was selected for BSA because of its good amplification power.

OPA36

Eleven bands were produced by OPA 36 (Plate 8a). Ten bands were clear and one band was faint. All the amplicons produced by this primer were monomorphic. Size of the amplicons produced by this primer ranged from 0.5 kb to 2 kb. This primer was selected for BSA because of its good amplification power.

OPA 38

OPA 38 produced a 0.9 kb amplicon in resistant genotype and susceptible genotype (Plate 8a). It failed to distinguish resistant parent from susceptible parent. This primer was selected for BSA because of its good amplification power.

OPA 39

OPA 39 produced six amplicons (Plate 8a). Five bands produced by this primer were clear and one band was faint. This primer exhibited zero percent polymorphism. Size of the amplicons produced by this primer ranged from 0.3 kb to 1.5 kb.

Among these primers OPA 04, OPA 6, OPA 9, OPA 24, OPA 26, OPA 34 and OPA 36 were selected for bulk segregant analysis.

4.3.4.1.3. Screening of OPB and OPC primers

Five primers from OPB and OPC series were used for screening Amplification of pattern OPB and OPC series primers given in (Table 12).

OPB 07

Screening of resistant and susceptible genotypes using OPB 07 produced five bands .One of the bands produced was clear and all other bands were faint. All the bands produced by this primer were monomorphic. Size of the bands produced ranged from 0.2 kb to 1 kb.

OPC 8

Four bands were produced by OPC 8 (Plate 9a). All the bands produced by this primer were clear and monomorphic. The size of the bands produced by this primer ranged from 0.5 kb to 2.1 kb. This primer couldn't produce a unique band in resistant genotypes.

OPC 9

Six bands were produced by OPC 9 (Plate 9a). All the bands produced were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.1 kb to 1.3 kb. This primer failed to produce a unique band in resistant genotypes.

Table 12. Amplification patterns of OPB and OPC primers

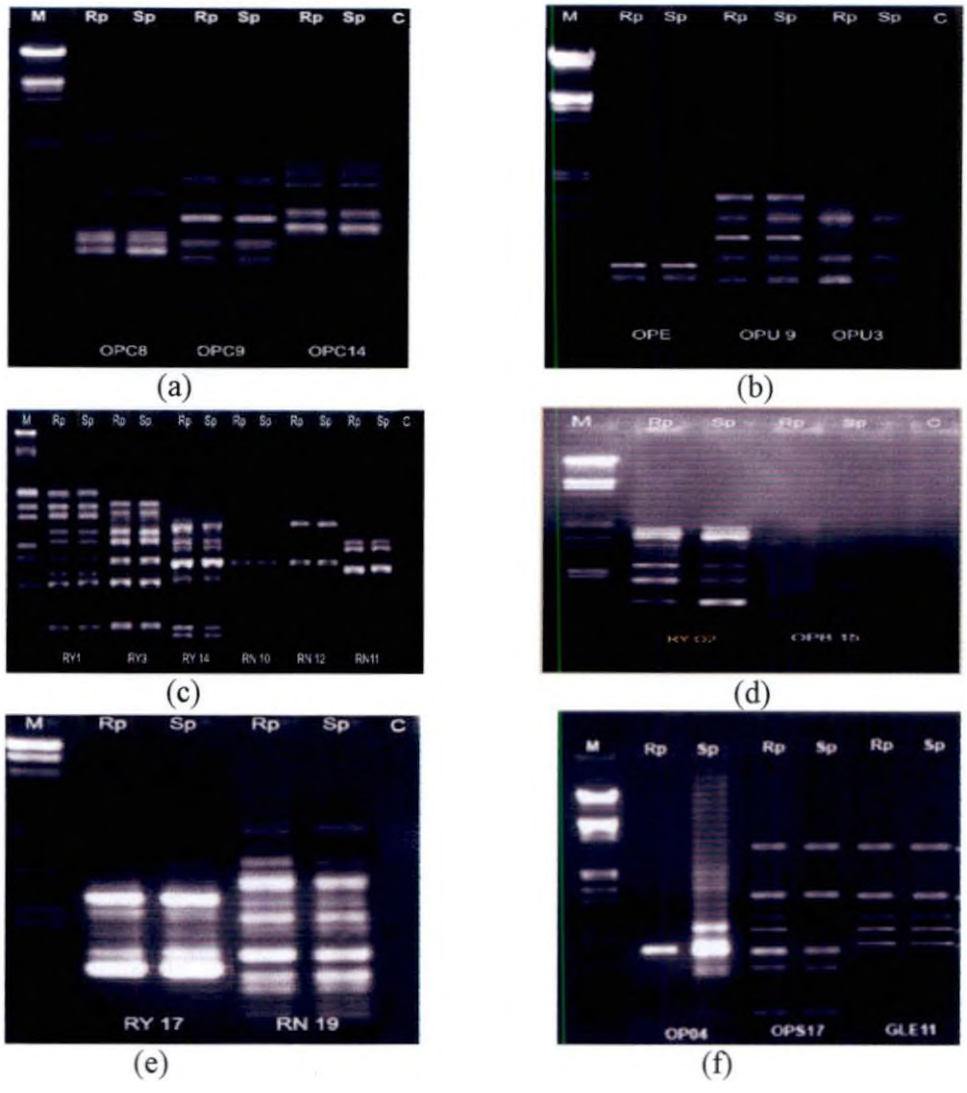
Sl.no	Primer	No of bands		% polymorphism	Amplification pattern
		SP	RP		
1	OPB 07	5	5	0	Average
2	OPC 8	4	4	0	Average
3	OPC 9	6	6	0	Good
4	OPC 14	5	5	0	Good

Table 13.Amplification patterns of OPE, OPF and OPU primers

Sl No	Primer	No of bands		% polymorphism	Amplification pattern
		SP	RP		
1	OPE	2	2	0	Poor
2	OPF 5	11	11	0	Good
3	OPU3	3	3	0	Average
4	OPU9	5	5	0	Good

Table 14. Amplification patterns of RN and RY primers

Sl No	Primer	No of bands		% polymorphism	Amplification pattern
		RP	SP		
1	RN 11	3	3	0	Poor
2	RN 12	2	2	0	Poor
3	RN 10	1	1	0	Good
4	RN 19	10	10	0	Good
5	RY 01	9	9	0	Good
6	RY 02	8	8	12	Good
7	RY 03	8	8	0	Good
8	RY 14	8	8	0	Good
9	RY 17	7	7	0	Good



M: λDNA/*Hind* III+ *Eco*RI double digest marker
RP: Resistant Parent
SP: Susceptible Parent C:Negative control
Primers-a)OPC 8,OPC 9,OPC 14 b)OPE,OPU 9,OPU 3 c)RY 1,RY 3,RY 14,RN 10,RN 12,RN 11 d)RY 02,OPB 15 e)RY 17,RN 19 f)OPA 04,OPS 17,GLE 11

Plate 9. Screening of brinjal genotypes using OPC, OPU, OPE,RN,OPB,OPS,GLE 11 and RY series of primers

OPC 14

Five bands were produced by OPC 14 (Plate 9a). All the bands produced by this primer were clear and monomorphic. The size of the bands produced by this primer ranged from 0.8 kb to 2kb. This primer couldn't produce a unique band in resistant genotypes.

4.3.4.1.4 .Screening of OPE, OPF and OPU primers

Four primers from OPE, OPF and OPU series were used for screening Amplification pattern of OPE, OPF and OPU primers given in (Table 13).

OPE 1

Two bands of 0.8 kb and 0.9 kb were produced by OPE 1 on both the genotypes selected for primer screening (Plate 9b). This primer exhibited zero percent polymorphism.

OPF 5

Eleven bands were produced by OPF 5. Nine bands were clear and two bands were faint.All the bands were monomorphic (Plate 8a). Size of the bands produced by this primer ranged from 0.1 kb to 2 kb. This primer failed to produce a unique band in resistant genotypes. This primer was selected for BSA because it exhibited good amplification power.

OPU 3

Three bands were produced by OPU 3 (Plate). The bands were faint and monomorphic. Size of the amplicons produced by this primer ranged from 0.8kb to 1.3kb.

OPU 9

Five bands were produced by OPU 9 (Plate 9b). Two bands were clear and three bands were faint. Size of the amplicons produced by this primer ranged from 0.8 kb to 1.5 kb .This primer failed to produce a band specific to resistant genotypes.

Among these primers OPF 5 was taken for bulk segregant analysis.

4.3.4.1.5. Screening of RY and RN primers

Eight primers from RY and RN series were used for screening Amplification pattern of RY and RN primers given in (Table 14).

RY 01

Nine amplicons were produced by RY 01 (Plate 9c). All the bands produced were clear and monomorphic. Size of the amplicons produced ranged from 0.2 kb to 2kb. This primer was selected for BSA because of its good amplification power.

RY 02

Screening of DNA of resistant genotype Surya and susceptible genotype Pusa Purple Long produced eight amplicons (Plate 9d). Among these seven amplicons were clear and one amplicon was faint. It produced a polymorphic band of 1.2kb unique to resistant parent Surya. Size of amplicons produced by this primer ranged from 0.5 kb to 1.9 kb. This primer exhibited 13 % polymorphism and it was selected for BSA.

RY 03

Eight amplicons were produced by this primer (Plate 9c). Seven bands produced were clear and one was faint. All bands were monomorphic. Size of the amplicons produced by this primer ranged from 0.2 kb to 1.5 kb. This primer was selected for BSA because of its good amplification power..

RY 14

Eight amplicons were produced by RY 14 (Plate 9c). All the bands produced were clear and monomorphic. Size of the amplicons produced ranged from 0.2 kb to1.3 kb.

RY 17

Seven bands were produced by RY 17 (Plate 9e). All the bands were clear and monomorphic. Size of the amplicons produced by this primer was between 0.1 kb and 1.37 kb. This primer couldn't produce a band specific to resistant genotypes.

RN 10

RAPD analysis of resistant and susceptible genotypes with RN 10 produced a monomorphic band of 0.8 kb (Plate).

RN 11

Three amplicons were produced by RN 11 (Plate 9c). All the bands produced were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.8 kb to 1.2 kb

RN 12

Two monomorphic amplicons of 1.3 kb and 0.8 kb were produced by RN 12 (Plate9c).

RN 19

Ten amplicons were produced by RN 19 (Plate 9e). Seven bands were clear and three bands were faint. Size of amplicons produced by this primer ranged from 0.4 kb to 2.0 kb. This primer failed to produce a unique band for resistant genotypes. This primer was selected for BSA because of its good amplification power.

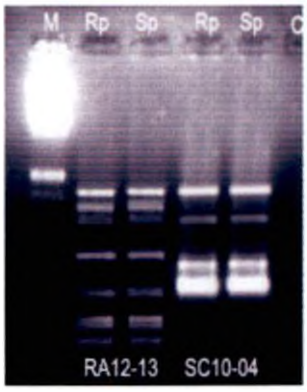
Among these primers RY 01, RY 02, RY 03 and RN 19 were taken for bulk segregant analysis.

4.3.4.1.6. Screening with wilt specific primers

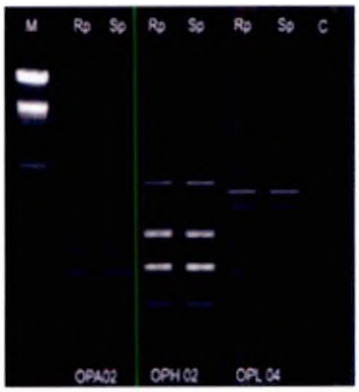
Thirty primers wilt specific primersc were used for screening. Amplification pattern of wllt specific primers given in (Table 15).

Table 15..Amplification patterns of wilt specific primers

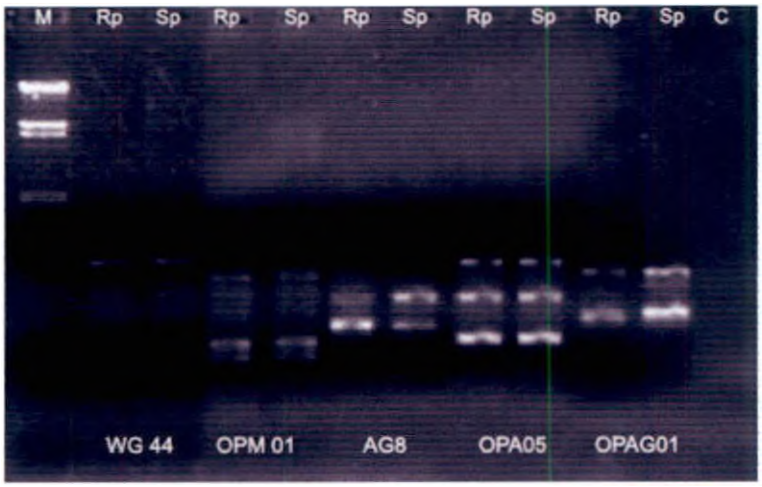
Sl No	Primer	No of bands		% polymorphism	Amplification pattern
		RP	SP		
1.	AG 8	3	3	0	Average
2.	WG 44	3	3	0	Average
3.	GLE 11	5	5	0	Good
4.	OPAG 01	2	2	0	Poor
5.	OPAH 12	5	5	0	Good
6.	OPAH 09	0	0	0	Poor
7.	OPH 02	6	6	0	Good
8.	OPM 01	3	3	0	Average
9.	OPAG 01	2	2	0	Poor
10.	OPL 04	5	5	0	Good
11.	OPG 16	6	6	0	Good
12.	OPH 06	6	6	0	Good
13.	RA 12-29	4	4	0	Average
14.	RF	5	5	0	Good
15.	SC 10-04	4	4	0	Average
16.	RA 12-84	0	0	0	Poor
17.	OPH 12	5	5	0	Good
18.	RA 12-13	7	7	0	Good
19.	OPH 07	6	6	0	Good
20.	RA 10-22	7	7	0	Good
21.	RA 12-74	6	6	0	Good
22.	RA 12-41	7	7	0	Good
23.	R 10	6	6	0	Good
24.	RA 12-38	6	6	0	Good
25.	OPG 03	6	6	0	Good



(a)



(b)



(c)

M: λDNA/*Hind* III+ *Eco*RI double digest marker
RP: Resistant Parent
SP: Susceptible Parent, C:Negative control

Primers-a) RA 12-13, SC 10-04
b) OPA 02, OPH 02, OPL 04
c) WG 44, OPM 01, AG 8, OPA 05, OPAG 01

Plate 10. Screening of brinjal genotypes using OPA and wilt specific primers

AG8

Screening of resistant genotype and susceptible genotype with AG8 gave three amplicons (Plate 10c). Two of the bands produced by this primer were clear and one band was faint. Size of the amplicons produced by this primer was 0.5 kb to 1.5 kb. This primer failed to produce a unique band in resistant genotypes.

WG 44

Three bands were produced by WG 44 (Plate 10c). One bnad was clear and other two bands were faint. All the bands were monomorphic. Size of the bands ranged from 0.5 kb to 1.1 kb.

GLE 11

Five amplicons were produced by GLE11 (Plate 10c). All the five bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.2 kb to 3.4kb. This primer couldnot produce a band specific to resistant genotype.

OPAG 01

Two bands were produced by OPAG 01 (Plate 10c). Two bands were clear and monomorphic. Size of the bands ranged between 0.5 kb and 0.9 kb. This primer exhibited zero percent polymorphism.

OPAH 12

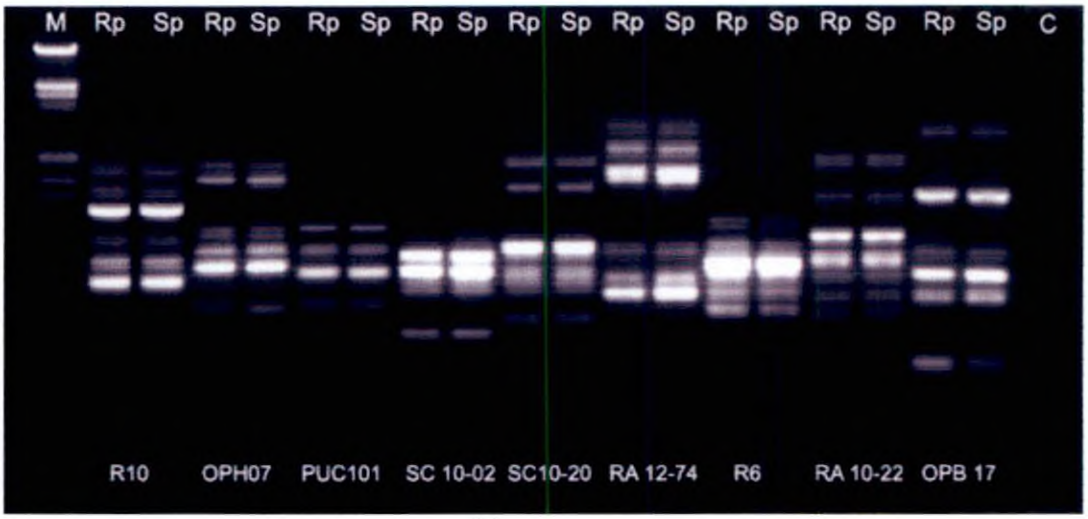
Five amplicons were produced by OPH 12 (Plate 11). All the 5 bands were clear and monomorphic. This primer couldn't produce a unique band in resistant genotypes. Size of the amplicons produced by this primer ranged from 0.4 kb to 1.3 kb.

OPH 02

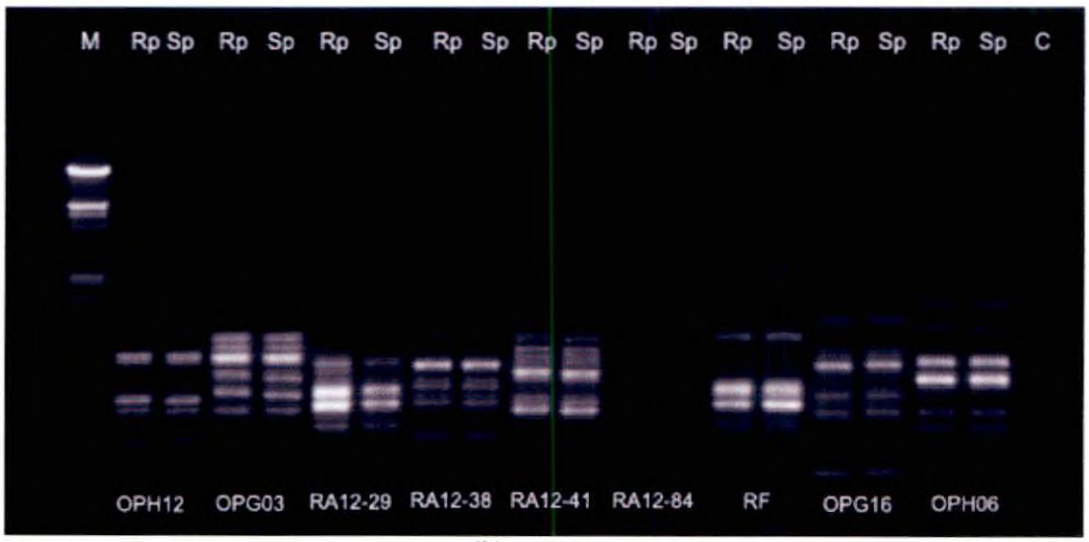
Six amplicons were produced by OPH 02 (Plate 10b). All bands were clear and monomorphic. Size of the amplicons produced ranged from 0.2kb to 1.9kb. This primer exhibited zero percent polymorphism.

Table. 15. Continued

26.	SC 10-20	6	6	0	Good
27.	SC 10-02	4	4	0	Average
28.	OPB 17	6	6	0	Good
29.	PUC 101	4	4	0	Average
30.	R 6	5	5	0	Good



(a)



(b)

M: λDNA/*Hind* III+ *Eco*RI double digest marker
RP: Resistant Parent
SP: Susceptible Parent, C:Negative control
Primers-a)R10, OPH 07, PUC 101, SC 10-02, RA 12-74, R6, RA 10-22, OPB 17 b)OPH 12, OPG 03, RA 12-29,RA 12-38,RA 12-41,RA 12-84,RF,OPG 16,OPH 08

Plate 11. Screening of brinjal genotypes using wilt specific primers

OPM 01

Three amplicons were produced by OPM 01 (Plate 10c). Three bands were faint and monomorphic. Size of the amplicons produced by this primer ranged from 0.4 kb to 1.2 kb. This primer couldn't produce a unique band in resistant genotypes.

OPL 04

Five amplicons were produced by OPL 04 (Plate 10b). All bands were faint and monomorphic. Size of the amplicons produced by this primer ranged from 0.6 kb to 2 kb. This primer couldn't produce a unique band in resistant genotypes.

OPG 16

Six amplicons were produced by OPG 16 (Plate 11b). All bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.3 kb to 1.3 kb. This primer couldn't produce a unique band in resistant genotypes.

OPH 06

Six amplicons were produced by OPH 06 (Plate 11b). All bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.5 kb to 1.5 kb. This primer couldn't produce a unique band in resistant genotypes.

RA 12-29

Four amplicons were produced by RA 12-29 (Plate 11b) .Three bands were clear and one band was faint. Size of the amplicons produced by this primer ranged from 0.8 kb to 0.9kb. This primer couldn't produce a unique band in resistant genotypes.

RF

Five bands were produced by RF (Plate b). Three bands were clear and two bands were faint. Size of the amplicons produced by this primer ranged from 0.83kb to 2 kb. This primer exhibited zero percentage polymorphism. This primer was selected for BSA because of its good amplification power.

SC 10-04

Four bands were produced by SC 10-04 (Plate 10a). The bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.56 kb to 2.0 kb. This primer couldn't produce a unique band in resistant genotypes.

RA 12-84

RA 12-84 didn't produce any amplification (Plate 11b).

OPH 12

Five bands were produced by OPH 12 (Plate 11b). All bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.2 kb to 1.3 kb. This primer exhibited zero percentage polymorphism.

RA 12-13

Seven bands were produced by RA 12-13 (Plate 10a). All the bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.1 kb to 1.5 kb. This primer exhibited zero percent polymorphism.

OPH 07

Six bands were produced by OPH 07 (Plate 11a). All bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.4 kb to 1.9 kb.This primer also couldn't produce a unique band in resistant genotypes.

RA 10-22

Seven bands were produced by RA 10-22 (Plate 11a). Four bands were clear and three bands were faint. Size of the amplicons produced by this primer ranged from 0.4 kb to 3 kb. This primer also exhibited zero percent polymorphism.

RA 12-74

Six bands were produced by RA 12-74 (Plate 11a). Four bands were clear and two bands were faint. Size of the amplicons produced by this primer ranged from 0.5 kb to 2.5 kb. This primer couldn't produce a unique band in resistant genotypes.

RA 12-41

Seven bands were produced by RA 12-41 (Plate 11b). Six bands were clear and one band was faint. Size of the amplicons produced by this primer ranged from 0.5 kb to 1.3 kb. This primer exhibited zero percent polymorphism. This primer was selected for BSA because of its good amplification power.

R10

Six bands were produced by R10 (Plate 11a). Three bands were clear and three bands were faint. Size of the amplicons produced by this primer ranged from 0.5 kb to 1.7 kb. This primer couldn't produce a unique band in resistant genotype.

RA12-38

Six bands were produced by RA 12-38 (Plate 11b). Four bands were clear and two bands were faint. Size of the amplicons produced by this primer ranged from 0.3 kb to 1.4 kb. This primer exhibited zero percent polymorphism.

OPG 03

Six amplicons were produced by OPG 03 (Plate 11b). All the bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.3 kb to 1.2 kb. This primer couldn't produce a unique band in resistant genotypes.

SC 10-20

Six bands were produced by SC 10-20 (Plate 11a). All bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.4 kb to 1.9 kb.

SC 10-02

Four bands were produced by SC 10-02 (Plate 11a). Three bands were clear and one band was faint. Size of the amplicons produced by this primer ranged from 0.2 kb to 0.8 kb. This primer couldn't produce a unique band in resistant genotype.

OPB 17

Six bands were produced by OPB 17 (Plate 11a). Five bands were clear and one band was faint. All the bands were monomorphic. Size of the amplicons produced by this primer ranged from 0.2 kb to 2.7 kb. This primer exhibited zero per cent polymorphism. This primer was selected for BSA because of its good amplification power.

PUC 101

Four bands were produced by PUC 101 (Plate 11a). All bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.1 kb to 2.7 kb. This primer was selected for BSA because of its good amplification power.

R6

Five bands were produced by R6 (Plate 11a). All bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from to 0.4 kb to 1.2 kb.

Among these OPL 04, RA 12-41, RF, OPB 17, PUC 101 were used for bulk segregant analysis.

4.3.4.2. Bulk segregant analysis (BSA) using selected RAPD primers

In primer screening, primers producing polymorphism was very less. Twenty two primers were selected for bulk segregant analysis. Among these three primers showed polymorphism and nineteen primers produced an average of five bands. List of primers selected for BSA are given in Table 16.

Bulk segregant analysis with primers which showed polymorphism

The primers RY 02, OPS 17 and OPA 04 showed polymorphism during primer screening. The banding pattern exhibited by these primers in BSA is discussed below.

Table 16.List of primers used for bulk segregant analysis

Sl No	Primer	No of bands		% polymorphism
		RP	SP	
1	RY 01	9	9	0
2	RY 02	8	7	12
3	RY 03	8	8	0
4	RN 19	10	10	0
5	OPS 4	10	10	0
6	OPS 9	7	7	0
7	OPS 10	10	10	0
8	OPS 16	10	10	0
9	OPS 17	7	5	29
10	OPA 04	1	5	80
11	OPA 6	8	8	0
12	OPA 9	7	7	0
13	OPA 24	5	5	0
14	OPA 26	10	10	0
15	OPA 34	10	10	0
16	OPA 36	11	11	0
17	OPL 04	5	5	0
18	OPF 5	9	9	0
19	RA 12-41	6	6	0
20	RF	5	5	0
21	OPB 17	5	5	0
22	PUC 101	5	5	0

RY 02

In primer screening RY 02 had produced 8 and 7 amplicons in resistant and susceptible parent respectively. In resistant parent out of the 8, one band of 1.2 kb was polymorphic. In BSA also this 1.2kb polymorphic band was present in resistant parent but absent in resistant bulk, susceptible parent, susceptible bulk and resistant variety Swetha. Hence the primer is incapable of distinguishing susceptible and resistant genotypes. In BSA also this primer exhibited original resolving power of 7-8 amplicons ranging from 0.5kb to 1.9 kb (Plate 12b)

The polymorphic ability of RY 02 was again checked in individual F2 plants of the bulk (Plate 13) .In this analysis also the polymorphic band of 1.2 kb was absent in resistant 5 F_2 plants.

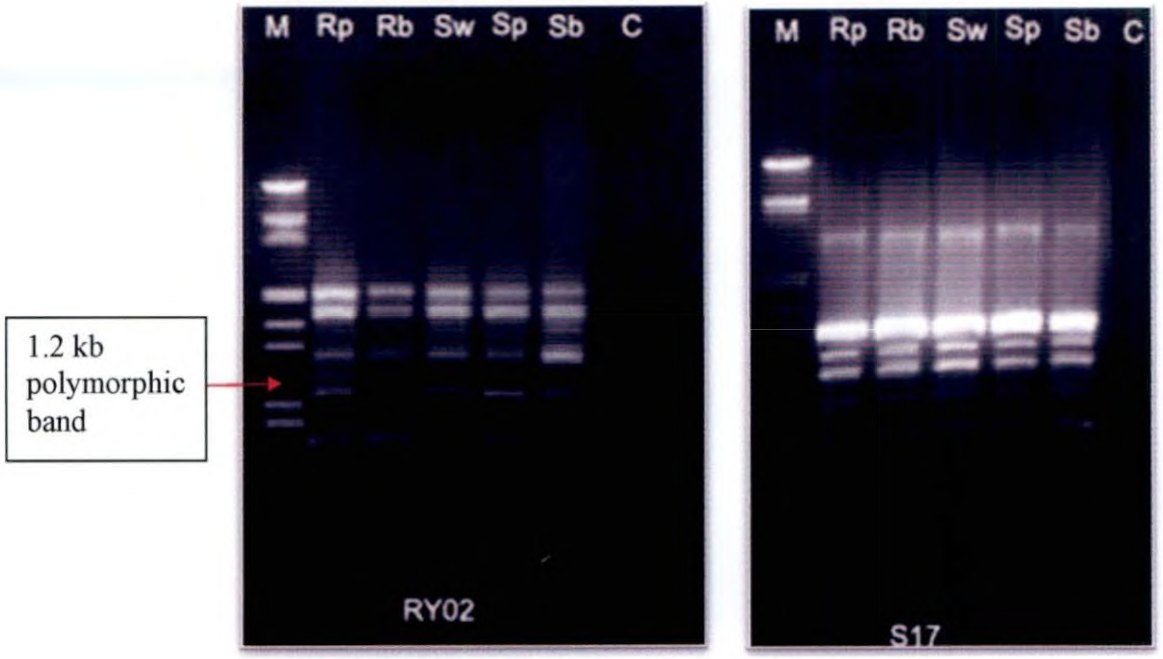
OPS 17

OPS 17 had produced 7 amplicons in resistant parent and 5amplicons in susceptible parent with band size ranging from 0.3kb to 3.4kb (Plate 12a). In the resistant parent two polymorphic amplicons of 1.1kb and 1.2 kb were present. But in BSA the polymorphic bands observed in resistant parent was present in all the genotypes selected. So the polymorphic amplicons were not linked with resistant character.

OPA 04

In primer screening OPA 04 has given only an amplicon in resistant parent and five amplicons in susceptible parent with a size ranging from 0.3Kbp to 2.0kb.In BSA OPA 04 has produced five amplicons ranging from to 0.3kbto 2.0kb.But all the bands were monomorphic(Plate 14c).

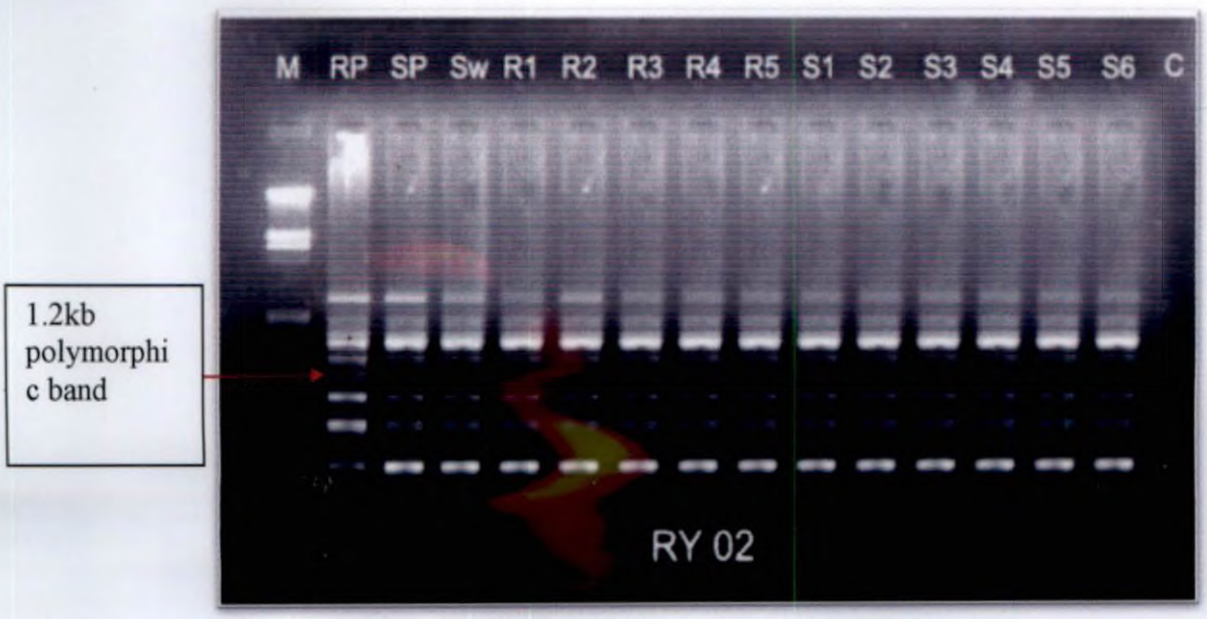
BSA with primers having good resolving power



(a) (b)

M: λDNA/*Hind* III+ *Eco*RI double digest marker ,
Rp: Resistant Parent
Rb: Resistant Bulk
Sp: Susceptible Parent
Sb: Susceptible Bulk
Sw-Swetha
C:Negative control

Plate 12.Bulk segregant analysis of brinjal genotypes with RY 02 and OPS 17 primers



M: λDNA/*Hind* III+ *Eco*RI double digest marker
Rp: Resistant Parent
Rb: Resistant Bulk
Sp: Susceptible Parent
Sb: Susceptible Bulk,
Sw-Swetha
R1-R5-Resistant F2 plants
S1-S5-Susceptible F2 plants
C:Negative control

Plate 13. Banding pattern of Surya,PPL,Swetha and F2 plants with primer RY 02

RY 01

RY 01 primer was selected for bulk segregant analysis because it produced good amplification pattern (Plate 15b). Nine amplicons were produced by this primer. All the bands produced were clear and monomorphic. Size of the amplicons produced ranged from 0.2 kb to 2 kb.

RY 03

RY 03 was selected for bulk segregant analysis because it produced good amplification pattern in primer screening. Seven amplicons were produced by this primer (Plate 15b). All the bands produced were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.2 kb to 1.5 kb.

RN 19

RN 19 was selected for bulk segregant analysis because it produced good amplification pattern.Nine amplicons were produced by this primer (Plate 15d). All bands were clear and distinct. Size of amplicons produced by this primer ranged from 0.4 kb to 2.0 kb. This primer failed to produce a unique band for resistant genotypes.

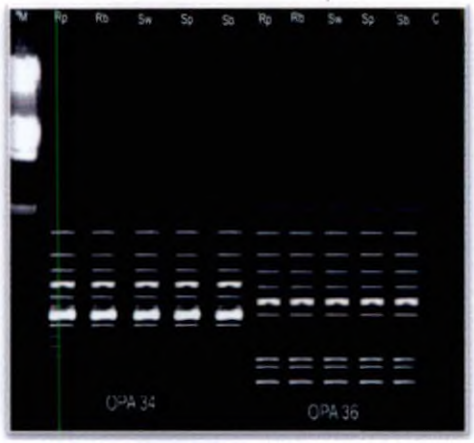
OPS 4

OPS 4 was selected for bulk segregant analysis because it exhibited good amplification during primer screening. In bulk also it exhibited good resolving power but all the bands produced were monomorphic(Plate 14 a). This primer produced 10 amplicons. Seven bands produced by this primer were clear and three bands were faint. Band width ranged from 0.4 kb to 2.1kb.This primer also produced zero percent polymorphism.

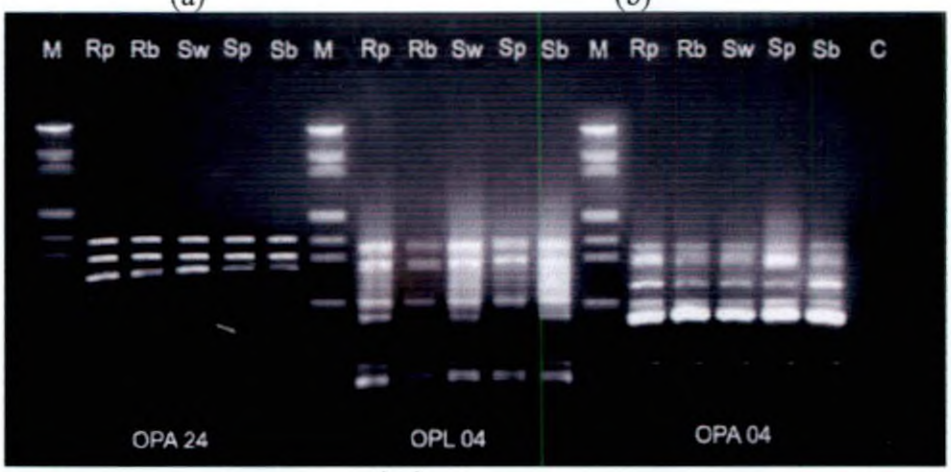
OPS 9

OPS 9 was selected for bulk segregant analysis because it exhibited good amplification during primer screening. Eight amplicons were produced by this primer (Plate 15a). Seven bands produced by this primer were clear and one band was faint.

(a)



(b)



(c)

M: λDNA/*Hind* III+ *Eco*RI double digest marker ,
Rp: Resistant Parent
Rb: Resistant Bulk
Sp: Susceptible Parent
Sb: Susceptible Bulk
Sw-Swetha
C:Negative control

Primers-a)OPS 4, b)OPA 34,OPA 36, c)OPA 24, OPL 04, OPA 04

Plate 14. Bulk segregant analysis with OPS 4,OPA 34,OPA 36,OPA 24,OPL 04,OPA 04

Size of the band produced by this primer was between 0.3 kb to 2 kb. This primer couldn't produce a unique band in resistant genotypes.

OPS 10

OPS 10 was selected for bulk segregant analysis because it exhibited good amplification during primer screening. Ten amplicons were produced by this primer (Plate 15c). All the bands produced by this primer were monomorphic. It couldn't produce a band unique to resistant parent Surya. The size of the bands produced by this primer ranged from0.1 kb to 2.1 kb. All the bands produced by this primr were clear.

OPS 16

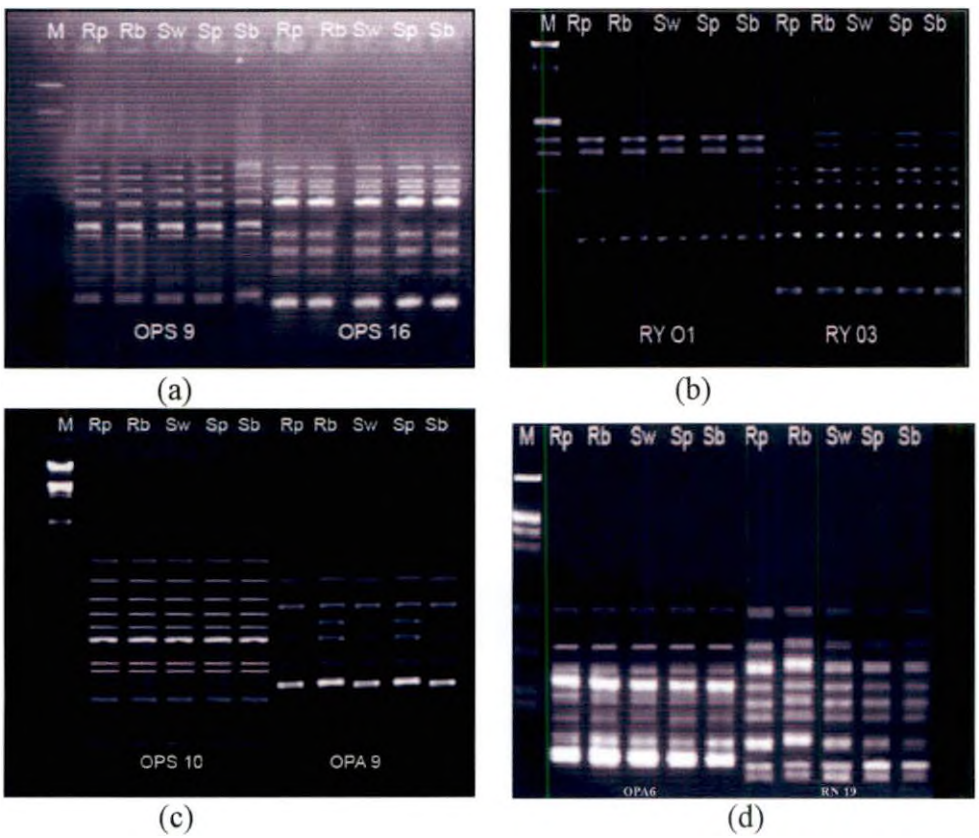
OPS 16 was selected for bulk segregant analysis because it exhibited good amplification during primer screening .Nine bands were produced by this primer (Plate 15a). Four bands were clear and five bands were faint. Band size produced by this primer ranged from 0.2 kb to 2.1 kb. This primer couldn't produce a unique band in resistant genotypes.

OPA 06

This primer was selected for bulk segregant analysis because it exhibited good amplification during primer screening. Nine bands were produced by this primer (Plate 15d). All the bands were clear. This primer exhibited zero percent polymorphism. Size of bands produced by this primer ranged from0.3 kb to 1.3 kb. .This primer couldn't produce a unique band to distinguish resistant genotypes.

OPA 9

This primer was selected for bulk segregant analysis because it exhibited good amplification during primer screening. Seven bands were produced by this primer (Plate 15c). All the bands were clear. The size of the bands produced by this primer ranged from 0.2 kb to 1 kb .This primer couldn't distinguish resistant genotypes from susceptible genotypes.



M: λDNA/*Hind* III+ *Eco*RI double digest marker ,
Rp: Resistant Parent
Rb: Resistant Bulk
Sp: Susceptible Parent
Sb: Susceptible Bulk
Sw-Swetha
C:Negative control

Primers-a)OPS 9, OPS 16 b)RY 01,RY 03 C)OPS 10, OPA 9 d)OPA6, RN 19

Plate 15.Bulk segregant analysis with OPS 9, OPS 16, RY 01, RY 03, OPS 10,OPA 09,OPA 6,RN 19

OPA 24

This primer was selected for bulk segregant analysis because it exhibited good amplification during primer screening. Five bands were produced by this primer(Plate 15c). All the bands produced by this primer were clear and monomorphic. Size of the bands produced by this primer ranged between 0.6 kbto 1 kb.

OPA 26

This primer was selected for bulk segregant analysis because it exhibited good amplification during primer screening. Nine bands were produced by this primer (Plate 16a). All the bands were clear and monomorphic. Size of the bands produced by this primer ranged from 0.5 kb to 1.9 kb. This primer couldn't distinguish resistant genotypes from susceptible genotypes.

OPA 34

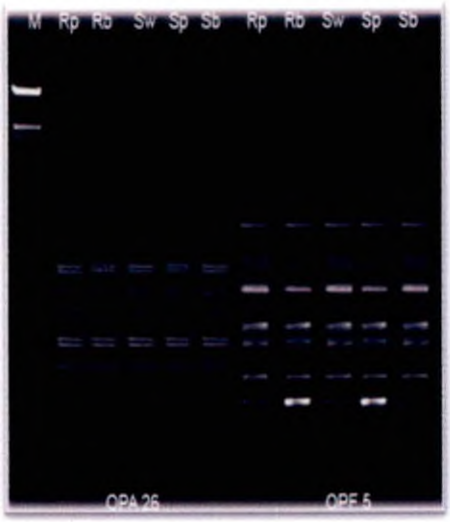
This primer was selected for bulk segregant analysis because it exhibited good amplification during primer screening. Eleven bands were produced by this primer (Plate 14b). Nine bands were clear and two bands were faint.Size of the amplicons produced by this primer ranged from 0.5 kb to 2 kb. This primer exhibited zero percent polymorphism. It couldn't produce a band specific to resistant genotypes.

OPA 36

This primer was selected for bulk segregant analysis because it exhibited good amplification during primer screening. Eleven bands were produced by this primer (Plate 14b). Ten bands were clear and one band was faint. All the amplicons produced by this primer were monomorphic. Size of the amplicons produced by this primer ranged from 0.5 kb to 2 kb.

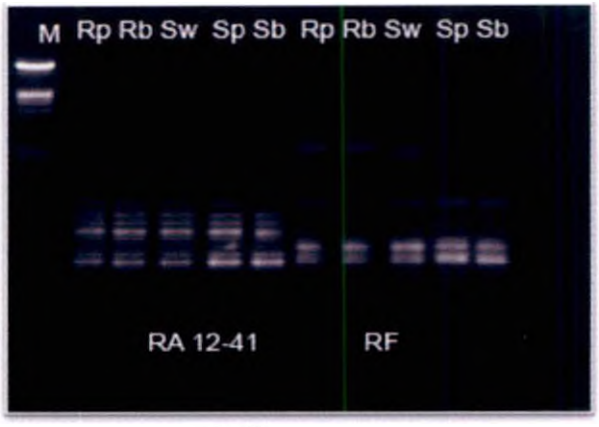
OPF 5

OPF 5 primer was used for bulk segregant analysis as it gave good amplification during primer screening. In BSA it gave very good amplicons (Plate 16a)



(a)

(b)



(c)

M: λDNA/*Hind* III+ *Eco*RI double digest marker ,
Rp: Resistant Parent
Rb: Resistant Bulk
Sp: Susceptible Parent
Sb: Susceptible Bulk
Sw-Swetha
C: Negative control
Primers-a) OPA 26, OPF 5 b)PUC 101, OPB 17, c)RA12-41, RF

Plate 16.BSA with OPA 26, OPF 5, PUC 101 ,OPB 17, RA 12-41 and RF

Eleven bands were produced by OPF. All the bands were monomorphic (Plate). Size of the bands produced by this primer ranged from 0.1 kb to 2 kb.

OPL 04

OPL 04 primer was used for bulk segregant analysis as it gave good amplification during primer screening. In BSA it gave very good amplification (Plate 14c). But all the bands produced were monomorphic. Five amplicons were produced by OPL 04. Size of the amplicons produced by this primer ranged from 0.6kb to 2kb .This primer couldn't produce a unique band in resistant genotype.

RA 12-41

Seven bands were produced by this primer. Six bands were clear and one band was faint (Plate 16c). Size of the amplicons produced by this primer ranged from 0.5 kb to 1.3 kb. This primer exhibited zero percent polymorphism.

RF

During primer screening RF gave good amplificarions. In BSA five bands were produced by RF. Three bands were clear and two bands were faint (Plate 16c).Size of the amplicons produced by this primer ranged from 0.83 kb to 2 kb. This primer exhibited zero percentage polymorphism.

OPB 17

Six bands were produced by OPB 17 in BSA (Plate 16b). Five bands were clear and one band was faint (Plate 16b). Size of the amplicons produced by this primer ranged from 0.3 kb to 2.9 kb.

PUC101

Five bands were produced by PUC 101 (Plate 16b). All bands were clear and monomorphic. Size of the amplicons produced by this primer ranged from 0.1 kb to 2.7 kb.

Discussion

5. DISCUSSION

Brinjal (*Solanum melongena* L.) occupies the third position amongst vegetable crops grown in India next to potato and tomato. Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* is one of the most serious diseases of crops in tropics, subtropics and warm temperate regions of the world. In India 75-81% losses reported in brinjal due to bacterial wilt (Singh, 1995). Warm, humid tropical climate and the acidic soil condition prevailing in Kerala favour the incidence of bacterial wilt.

As the application of chemicals, soil fumigation and crop rotation are practically ineffective, the use of resistant varieties is the most effective means for control of bacterial wilt. Conventional breeding involving germplasm collection, selection and heterosis breeding has resulted in the release of a number of resistant varieties with desirable agronomic traits. The Department of Olericulture, College of Horticulture has developed bacterial wilt resistant varieties like Surya, Swetha, Haritha and Neelima in brinjal (Gokulapalan *et al.*, 2004).

Knowledge on the inheritance of resistance is important in breeding for resistance. . Study on the nature of inheritance showed that bacterial wilt resistance is monogenic and homozygous recessive (Vijaygopal, 1969; Gopimony, 1983; Varghese, 1991). Many resistant genes had been defined as polygenic (Acosta *et al.*, 1964; Gilbert *et al.*, 1974; Thurston, 1976; Yue *et al.*, 1995; Osiru *et al.*, 2001). But the mode of inheritance is still uncertain and the resistance is strongly influenced by environmental conditions such as temperature, pH and moisture content of soil.

Molecular marker offers a great potential in breeding for disease and pest resistance as they can be employed in selection of genotypes with specific character. Molecular markers such as RAPD,RFLP,SSR,AFLP,SCAR,STS and ISSR using F_2 and back cross populations NIL's, RIL's and doubled haploids are used for this.

Progress has been made in mapping and tagging many agriculturally important genes with molecular markers, which forms the foundation for marker-assisted selection. Molecular

markers have several advantages over the traditional phenotypic markers that were previously available to plant breeders. It gives accurate information about the susceptibility/resistance of the genotypes to a particular disease at an early stage of plant growth compared to field screening with artificial inoculation. It also eliminates environmental influence in resistance/susceptibility reaction of genotypes in field screening.

Availability of molecular markers linked to resistance genes will help in identifying plants carrying these genes in various generations without subjecting them to pathogen infection. When reliable markers are identified and gene is tagged with them they would prove to be a very powerful tool especially in screening and selection for quantitative and qualitative characters there by reducing the burden of plant breeders to a great extend. . Using recombinant DNA technology, desirable cloned genes could be transferred to desirable agronomic background within a shorter time cutting across species boundaries.

Surya (SM 6-7) with purple, oval fruits is a national variety with a productivity 30 t/ha . It was developed by single plant selection from an Annamalai collection SM 6. Swetha (SM 6-6) with white elongated and small fruits was also developed from SM 6 by single plant selection with a productivity 30 t/ha (Gokulapalan *et al.*, 2004).Both these varieties can be source of resistance for bacterial wilt resistance in crop improvement programme .Hence the present study "Development of a molecular marker for brinjal (*Solanum melongena* L.) varieties Surya and Swetha " was taken up. The methodology involved was bulk segregant analysis. The technical programme involved the following aspects:

1. Development of segregating F_2 generation for the trait under consideration
2. Phenotyping of genotypes for bacterial wilt resistance
3. Molecular characterization of brinjal genotypes with RAPD marker for getting polymorphism between resistant and susceptible genotypes

The results obtained on various aspects are discussed here under.

5.1. DEVELOPMENT OF SEGREGATING F_2 GENERATION FOR TRAIT UNDER CONSIDERATION

Controlled crosses were made using the resistant variety Surya as the ovule parent and susceptible variety Pusa Purple Long as the pollen parent, and F_1 plants were generated (Plate 2c). Thirty five F_2 plants (Plate 2d) were raised from a single F_1 plant by selfing and they were used as segregating population for tagging the bacterial wilt resistance gene. Segregating population for the character under consideration is necessary to know the gene expression under homozygous dominant, homozygous recessive and heterozygous condition. It will reveal monogenic or polygenic nature of the character. F_2 plants offer certain advantages over other mapping populations (DH lines, Nil's etc) because segregation is fixed (Benjamin and Burr, 1999).

The usual method to locate and compare loci regulating quantitative traits (QTL's) requires a segregating population of plants with each one genotyped with molecular markers. However plants from such segregating population are grouped according to phenotypic expression of trait and tested for differences in allele frequency between the population bulks (Bi-hao *et al.*, 2009).

5.2. PHENOTYPING OF GENOTYPES FOR BACTERIAL WILT RESISTANCE

5.2.1. Standardisation of artificial inoculation technique

The genotypes used were the susceptible variety Pusa Purple Long, resistant variety Surya and F_1 plants. Artificial inoculation was given to 30 days old seedlings at 3-4 leaf stage with fresh bacterial ooze. Two methods of inoculation *viz.*, stem puncturing and soil drenching with wounding were compared. Among the two methods evaluated, maximum wilt incidence was observed for stem puncturing method (90%) within a period of 15 days. The susceptible genotypes showed wilting within five days in stem puncture method while it was eight days in soil drenching with wounding.

Winstead and Kelman (1952) had reported about the efficiency of stem puncture method for artificial inoculation in tomato. The stem inoculation technique helped in rapid development

of disease symptoms as there was direct introduction of pathogen into the vascular tissues, which enabled faster movement and multiplication of the pathogen. James (2001) also made similar observations and reported stem puncturing in tomato to induce 100 percent wilt incidence one week after inoculation. Karumannil (2007) and Ragina (2009) also used stem puncturing method to induce wilt incidence in tomato seedlings. Moreover sufficient inoculum was ensured by keeping a piece of cotton dipped in bacterial suspension in the punctured leaf axils. Initial wilting symptom in inoculated seedlings was observed within five days after inoculation.

Being younger (30 days old), the seedling succumbed to wilt more rapidly. The reason for this could be that the seedlings have thinner cortical cells compared to older plants that make the entry of pathogen easier. Winstead and Kelman(1952) and Celine (1981) reported that wilting was more in juvenile stage as compared to one observed in adult stage.

Stem puncture method was selected for phenotyping of genotypes for bacterial wilt resistance because it showed early wilting and more percentage of wilting.

5.2.2. Phenotyping of genotypes for bacterial wilt incidence

Resistant parent Surya, susceptible parent Pusa Purple Long, F_1 and F_2 of cross Surya x Pusa Purple Long were inoculated artificially by stem puncture method and scoring of genotypes as resistant/susceptible was done by Mew and Ho (1976) system. Within a period of one month susceptible plants succumbed to death. Observations revealed that Surya was resistant with 100 per cent survival and Pusa Purple Long was susceptible with zero per cent survival.F_1 and F_2 plants were susceptible with 10 % and 17 .% per cent survival respectively. According to Gopulapalan *et al.* (2004) Surya is resistant to bacterial wilt and it was observed in present study also.

Phenotyping of genotypes with artificial inoculation classified the varieties, and F_1 and F_2 progenies into various groups according to the degree of susceptibility/resistance reaction. It also threw light on the genetic basis of bacterial wilt resistance.

Varghese (1991) reported monogenic recessive gene action in brinjal for wilt resistance after evaluating six generations of cross 'Surya X Pant Rituraj'. In the present investigation F_2 showed only 17% resistant progenies. So the observation made by Varghese (1991) may be true. Monma and Sakata (1993) reported that the bacterial wilt resistance was partially recessive as there was incomplete dominance towards susceptibility. Geetha and Peter (1993) reported that F_1 hybrids in which only resistant parents were involved were resistant, while the hybrids in which susceptible genotype was one of the parents were either susceptible or moderately resistant showing recessive mode of inheritance of bacterial wilt resistance in brinjal. Studies conducted at Kerala Agricultural University, Vellanikkara in brinjal and chilli revealed that resistant F1s could be developed by crossing resistant parents which indicates recessive mode of inheritance of bacterial wilt resistance (KAU, 1989).The F_1 population in the present investigation showed 10 per cent resistant plants. This can be due to the interference of other factors which also control bacterial wilt resistance. The population used for the screening study was small to get conclusive results.

Kelman (1953) reported that resistance to *Pseudomonas solanacearum* in groundnut, tobacco and brinjal are controlled by polygenes. Suzuki *et al.* (1964) observed that the bacterial wilt resistance in brinjal varieties Taiwan Naga and OTB-1 is inherited as quantitative character controlled by a number of genes. Li *et al.* (1989) stated that in a few crosses in brinjal where the F_1 was midway between the parents, no apparent dominance was shown.

Singh (1961) reported that multiple recessive genes control the resistance to bacterial wilt in tomato where as Akiba *et al.* (1972) observed that it was governed by a pair of dominant genes. Reports from Asian Vegetable Research and Development Centre, Taiwan (AVRDC, 1975) also revealed that multiple recessive genes acting additively controlled resisatnce to bacterial wilt in tomato. Sreelathakumary (1983) reported that a complementary and hypostatic type of digenic recessive gene responsible for resistance to bacterial wilt in tomato. Rajan (1985) reported that the bacterial wilt resistance in tomato variety 'Sakthi' is monogenic and incompletely dominanat.

At Kerala Agricultural University, Girijadevi and Peter (1987) made crosses of two hot pepper lines viz., 'Manjai' and 'Pant C-1' with five sweet peppers. All the F1s were susceptible or moderately susceptible indicating the recessive nature of inheritance of resistance to bacterial wilt. Markose (1996) studied the inheritance of bacterial wilt resistance using resistant variety Ujwala and susceptible variety Pusa Jwala by developing six generations of P_1, P_2, F_1, F_2, B_1 and B_2. Performance of the six generations showed that the resistance in Ujwala is monogenically inherited and is incompletely dominant.

For tagging bacterial wilt resistance gene, contrasting genotypes for the character of concern ie. bacterial wilt resistance are needed. The F_2 population used in the study was developed from the parents showing maximum and minimum bacterial wilt per cent which is the most effective and excellent method for developing linked markers .The DNA of thirty five F_2 plants tested were isolated prior to the artificial inoculation. DNA of five susceptible and resistant genotypes for bulking were selected after obtaining the screening data.

5.3. MOLECULAR CHARACTERISATION OF BRINJAL GENOTYPES WITH RAPD MARKER

Molecular characterization of the brinjal genotypes selected for the study was carried out with the objective of developing trait specific marker for bacterial wilt resistance. RAPD (Random Amplified Polymorphic DNA) assay was used for characterization in parent genotypes, resistant bulk, susceptible bulk and Swetha.

5.3.1. Genomic DNA used for molecular characterization

Isolation of good quality genomic DNA is one of the most important prerequisites for doing RAPD analysis. The procedures reported by Doyle and Doyle (1987) and Rogers and Bendich (1994) for the extraction of nucleic acids were compared for the isolation of genomic DNA from brinjal with slight modifications. Tender leaves were chosen for DNA extraction in all the cases .The method suggested by Rogers and Bendich (1994) was the best for the isolation

of the genomic DNA from brinjal since distinct bands without shearing were obtained. Recovery of DNA was also high.

Babu (2000) reported that the quality and quantity of DNA isolated was best when tender leaves were used as compared to mature and half mature leaf samples. Tender leaves contain actively dividing cells with lesser concentration of extra nuclear materials like protein, oil, carbohydrates, fats and other metabolites that interfere with the isolation of nucleic acids. Fu *et al.* (2003) also repoted that the use of young leaves was best for DNA isolation for RAPD assay.

The DNA isolated using the Rogers and Bendich (1994) protocol was appeared as clear and distinct band with less RNA contamination in the agarose gel compared with that of Doyle and Doyle (1987) method (Plate) Micheli *et al.* (1994) reported that RNA in the genomic DNA preparation often influences the reproducibility of RAPD patterns. So, an attempt was made to remove the contaminants by treatment with Ribonuclease A. Intact DNA bands were obtained after treatment (Plate).

The quality and quantity of DNA thus isolated was analyzed using both electrophoretic and spectrophotometric methods (Nanodrop ® ND-1000 spectrophotometer). In all cases intact clear narrow band indicated non-degraded DNA.The absorbance of nucleic acid samples were measured at a wavelength of 260nm and 280nm. The purity of DNA was assessed by the ratio OD 260/OD 280. The ratio of absorbance ranged between 1.80-1.90 which indicated that quality of DNA was good (Table). The DNA ,thus isolated after an appropriate dilution was used as template for RAPD analysis.

5.3.2. RAPD (Random Amplified Polymorphic DNA) analysis

The RAPD technique was developed by Williams *et al.* (1991) .This technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary oligonucleotide primers. In RAPD markers, polymorphism results from changes in either the sequence of the primer binding site (which prevents stable association with the primer) or from mutations like insertion, deletion, inversion etc (which alter the size or prevent the successful

amplification of a target DNA). This procedure is rapid, requires only small amounts of DNA, which need not be of high quality and involves no radioactivity. The information content in an individual RAPD marker is very low. It is only when many of these anonymous markers are used to define a genome that they begin to have utility (Williams *et al.*, 1990).

Williams *et al.* (1990) reported that even a single base change in the primer sequence could cause a complete change in the set of amplified DNA segments. They also found that the GC content in the primer influenced the amplification and a GC content of 40 per cent or more in the primer sequence was needed to generate detectable levels of amplified products. Welsh and McClelland (1990) observed that primers of similar length but different sequence gave a different set pattern since the template-primer interactions were different. Fakuoka *et al* (1992) reported that in rice increased GC content of the primers in the range of 40-60 per cent tended to increase the number of amplification products.

Usually RAPD markers are dominant in nature (Waugh and Powell, 1992) because polymorphisms are detected as the presence or absence of bands. Johnson *et al.* (1997) reported molecular tagging of the recessive bc-3 gene which confers resistance to bean common mosaic virus using RAPD technique in Andean common bean. Gangashetti *et al* .(2006) reported inheritance of elongated uppermost internode and identification of RAPD marker linked to recessive *eui* gene in rice.In Surya and Swetha bacterial wilt resistance is homozygous recessive in nature. The RAPD marker was selected for tagging the bacterial wilt resistance gene as the procedure is rapid requires only small amounts of DNA and involves no radioactivity. The reproducibility of RAPD marker can be improved by converting it to SCAR or STS.

5.3.2.1. Screening of primers for RAPD analysis

Ninty two decamer primers in the series of OPA, OPB, OPC, OPF, OPE, OPU, OPH, OPAH, OPAG, OPL, OPM, RY, RN, RA, SC, RF, AG 8, WG 44, GLE11, RF, R10, R6, and PUC101 as shown in (Table 2 to Table 7) were screened for DNA amplification. Among them thirty were wilt specific primers. These primers produced good amplifications but none of them

produced polymorphism.The template DNA were of resistant variety Surya and susceptible variety Pusa Purple Long.

Twenty two primers were selected for molecular characterization of selected genotypes through bulk segregant analysis based on presence of polymorphism and presence of five or more bands. Among the primers used for screening RY 02, OPA 04 and OPS 17 exhibited polymorphism. So these primers along with 19 other primers which gave five or more than five bands were selected for bulk segregant analysis. Screening of those primers with maximum amplification of DNA of resistant and susceptible genotypes minimized the labour and input required for total RAPD analysis. Sharma *et al.* (2004) and Uma *et al.* (2004) have used random primers from different Operon primer series in the RAPD studies.

5.3.2.2. Bulk Segregant Analysis (BSA)

Bulked Segregant Analysis was carried out with resistant parent (Surya), susceptible parent (Pusa Purple Long), resistant bulk (5 resistant F_2 plants), susceptible bulk (5 susceptible F_2 plants) and Swetha. Resistant bulk and susceptible bulk consisted of 5 each resistant and susceptible F_2 plants originated from a single F_1 by selfing. The resistant and susceptible F_2 were identified by phenotyping of F_2 plants after artificial inoculation with bacterial ooze. Before inoculation DNA of these plants were isolated.

Twent two primers, selected after primer screening ,belonging to OPA, OPB, OPS, OPF, RY,RN,RA, RF, PUC were used for bulk segregant analysis. Out of the 22 selected primers screened for polymorphism , RY 02 produced a polymorphic band of size 1.2 Kbp in resistant parent Surya. But it was absent in resistant bulk and Swetha. Banding pattern of individual F_2 plants with RY 02 was also checked. In these plants also 1.2Kbp polymorphic band was absent.

Bulked segregant analysis was done to identify RAPD markers linked to a gene with major effects for bacterial wilt resistance.BSA involves comparing two pooled DNA samples of individuals from a segregating population originating from a single cross (Michelmore *et al.*, 1991).Within each pool or bulk, the individuals are identical for the trait or gene of interest but

are arbitrary for all other genes.BSA removes the need for screening the entire mapping population with every primer. The minimum size of the bulk is determined by the frequency with which linked loci might be detected as polymorphic between the bulked samples.BSA has also been used to screen specific traits with RFLP also (Monna *et al.*, 1995 ; Chague *et al.*, 1996).

In primer screening and BSA, RAPD primers were not able to distinguish resistant and susceptible genotypes. Narrow level of polymorphism at molecular level of *Solanum* spp. may be the reason for it. Karihaloo *et al.* (1995) by their RAPD analysis carried out on 52 accessions of *S. melongena* and related weedy form "insanum" reported that even though *S.melongena* and insanum are highly diverse morphologically , they were not distinguishable.

Clain *et al.* (2004) reported homogeneity between accessions of *Solanum torvum* regarding high bacterial wilt tolerance. It was based on the genetic homogeneity (zero percent polymorphisms) revealed by 168 RAPD primers. Archak *et al.* (2002) analysed the genetic diversity of 27 tomato cultivars grown in India with RAPD markers generated with 42 random primers and reported low levels of polymorphism in tomato cultivars by RAPD. They reported difficulties for molecular characterization of cultivars of diploid autogamous solanaceous species. Gang *et al.* (2002) and Karumannil (2007) also reported low level of polymorphism at molecular level for *Solanum* spp.

Since bulk segregant analysis with 22 RAPD primers didn't give polymorphism between resistant and susceptible genotypes no RAPD marker linked with bacterial wilt resistance was identified. In primer screening out of the 92 primers screened, three had given polymorphism between resistant parent and susceptible parent. But BSA has showed that it is not linked with bacterial wilt resistance. Some of the genome analysis with RAPD reviewed above reveal that polymorphism in *Solanum* at DNA or molecular level is low. Genome analysis with more number of RAPD/ISSR primers may result in polymorphism between resistant and susceptible genotypes.

5.4. FUTURE LINE OF WORK

1. Molecular characterization of susceptible and resistant bulk with more number of primers to get polymorphism

2. Molecular characterization can be done with co-dominant markers like SSR and RFLP which would distinguish homozygous dominant from heterozygous dominant

3. The genetic makeup of the susceptible parent can be checked for homozygous/heterozygous condition in relation to bacterial wilt resistance by phenotyping of selfed progenies.

4. Isolate mRNA from resistant genotypes after artificial inoculation with pathogen, followed by cDNA synthesis and genome analysis

Summary

6. SUMMARY

The study entitled "Development of a molecular marker for brinjal (*Solanum melongena* L.) varieties Surya and Swetha" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB),College of Horticulture, Vellanikkara during the period 2009-2011.The main objective of the study was to develop a molecular marker for bacterial wilt resistance in the aforesaid varieties of brinjal with RAPD assay. The genotypes used for the study were resistant varieties Surya, Swetha , susceptible variety Pusa Purple Long and F_2 progenies of the cross Surya x Pusa Purple Long.

The salient findings of the study are summarized below:

1. F_1 plants were raised by controlled crossing of resistant variety Surya as the ovule parent and susceptible variety Pusa Purple Long as the pollen parent. Single F_1 plant was selfed and segregating F_2 progenies for bacterial wilt resistance were raised.

2. Two different methods *viz.*, stem puncturing and soil drenching with root wounding were compared for delivery of inoculum of *R.solanacearum* for bacterial wilt incidence. In soil drenching with root wounding method wilting percent was 75 within a period of 30 days. Stem puncturing method recorded 90 per cent wilting within 15 days. So stem puncturing method was used for phenotyping of genotypes for bacterial wilt incidence.

3. The genotypes-Surya, Pusa Purple Long,F1 and F2 of cross Surya x Pusa Purple Long were phenotyped after artificial inoculation and classified into resistant or susceptible according to Mew and Ho (1976) system. Surya was ranked as resistant with 100 per cent survival and Pusa Purple Long as susceptible with 0 per cent survival. In F_1 and F_2 the survival percentage was 10 and 17 respectively and were ranked as susceptible.

4. The protocols suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were compared for the isolation of genomic DNA. Tender leaves from the selected plants were used for the genomic DNA isolation. The protocol suggested by Rogers and Bendich (1994) was found to be best for the isolation of genomic DNA from brinjal. The RNA contamination was completely removed through RNase treatment. The quantity and quality of DNA was analyzsed by both electrophoresis and spectrophotometric method (NanoDrop® spectrophotometer). Agarose gel electrophoresis revealed clear intact band without shearing. In spectrophotometer the absorbance ratio ranged from 1.80-2.0 which indicated good quality of DNA. In all RAPD assay good quality DNA of aforesaid absorbance ratio was used.

5. Primer screening was done to select primers with good DNA amplification and polymorphism between resistant and susceptible genotypes. Ninty two 10-12 bp primers belonging different series viz., OPA, OPB, OPC, OPF, OPE, OPU, OPH, OPAH, OPAG, OPL, OPM, RY, RN, RA, SC, RF, AG 8, WG 44, GLE11, RF, R10, R6, and PUC101 were used for primer screening. This included thirty wilt specific primers also. Primers were selected for bulk segregant analysis based on polymorphism and good DNA amplification. Among the primers used for screening RY 02, OPA 04 and OPS 17 exhibited polymorphism. So these primers along with 19 other primers which gave five or more than five bands were selected for bulk segregant analysis.

6. Bulk segregant analysis was done with DNA of resistant parent Surya, susceptible parent Pusa Purple Long, resistant variety Swetha , resistant and susceptible bulk. Out of the 22 primers used in bulk segregant analysis, none gave polymorphism between resistant parent, resistant bulk, Swetha, susceptible parent and susceptible bulk. The polymorphism generated by the primer RY 02 was rechecked by PCR amplification of individual susceptible and resistant F_2 plants. None of the resistant F_2 plants produced 1.2Kbp polymorphic band as seen Surya.

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* Originals not seen

Appendices

APPENDIX-1

LIST OF LABORATORY EQUIPMENTS USED FOR THE STUDY

Spectrophotometer	Spectronic Genesys-5, Spectronic Instrument USA
Refrigerated centrifuge	Kubota,Japan
Horizontal electrophoresis system	Biorad
Thermal cycler	Mastercycler personal,Eppendorf
Gel documentation system	BIO-RAD Imaging system

APPENDIX-II

COMPOSITIONS OF REAGENTS USED FOR DNA ISOLATION

1.Doyle and Doyle method

4X Extraction buffer
Sorbitol-2.5g
Tris HCl-4.8g
EDTA-0.74g
Dissolved in 80ml of distilled water,adjusted the pH to 7.5 and made upto 100ml with distilled water

Lysis buffer
Tris HCl(1M,pH 8)-20ml(15.76g per 100ml)
EDTA(0.2ml)-20ml(9.305g per 100ml)
NaCl(5M)-40ml(29.22 g per 100ml)
Distilled water-20ml
CTAB-2g(Dissolved in 20ml distilled water and then added to the remaining components).

5% Sarcosin
Sarcosin-5g dissolved in 100ml distilled water

TE buffer
10mM Tris(pH 8)
1mM EDTA(pH 8)

2.Rogers and Bendich (CTAB) method

2X CTAB extraction buffer
CTAB(2%,v/v)
100mM Tris buffer(pH 8)
20mM EDTA(pH 8)
1.4M NaCl
1 % PVP

10% CTAB solution
10% CTAB (w/v)
0.7M NaCl

TE buffer
10mM Tris(pH 8)
10mM EDTA (pH 8)

Appendix –III

COMPOSITON OF BUFFERS AND DYES USED FOR GEL ELECTROPHORESIS

1. TAE buffer 50X (for 1litre)
242p Tris base
57.1 ml glacial acetic acid
100 ml 0.5M EDTA

2. TBE buffer 10X (for 1 litre)
54g Tris base
27.5g Boric acid
20ml 0.5M EDTA (pH 8.0)

3. Loading Dye(6 X)
0.25% bromophenol blue
0.25% xylene cyanol
30 % glycerol in water

4. Formamide dye
Formamide-10ml
Xylene cyanol-10mg
Bromophenol blue-10mg
0.5M EDTA (pH-8.0)-200µl

Development of a molecular marker for bacterial wilt resistance in brinjal (*Solanum melongena* L.) varieties Surya and Swetha

By

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(2009-11-122)

ABSTRACT OF THE THESIS

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ABSTRACT

The study entitled 'Development of a molecular marker for bacterial wilt resistance in brinjal (*Solanum melongena* L.) varieties Surya and Swetha' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2009-2011.

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* is one of the important problems of brinjal cultivation in warm humid tropics. The loss due to this varies from 30-100 per cent. Use of chemicals and field sanitation are not sufficient for controlling the disease. Worldwide approach is to use resistant varieties. KAU has developed and released bacterial wilt resistant brinjal varieties for cultivation. The Surya and Swetha are two among them and have received bacterial wilt resistance from SM-6 an Annamalai collection.

This investigation was taken up to develop a molecular marker for bacterial wilt resistance in Surya by RAPD through bulk segregant analysis as reported by Michelmore *et al* (1991). It also aimed to test the suitability of the same for identifying bacterial wilt resistance trait of resistant variety Swetha. The genotypes used for the study were Surya, Pusa Purple Long (susceptible variety released from IARI), Swetha and F_2 population of the cross between Surya and Pusa Purple Long. To raise segregating F_2 population F_1 was raised by controlled crossing of Surya with pollen grains of Pusa Purple Long. Then F_1 plant was selfed to get F_2 population.

Two different methods *viz.*, stem puncturing and soil drenching with root wounding were compared for the delivery of inoculum of *R. solanacearum* for bacterial wilt incidence and stem puncture method was found as the best. So stem puncturing method was used for phenotyping of genotypes for bacterial wilt incidence.

The F_2 population along with Surya, Pusa Purple Long and F_1 were phenotyped for bacterial wilt incidence. This was done through artificial inoculation with *Ralstonia solanacearum* by stem puncture method. Confirmation was done by ooze test. The genotypes were classified according to classification of Mew and Ho (1976). The variety Surya was resistant and Pusa Purple Long was susceptible. F_1 population showed 90 per cent susceptibility while F_2 population showed 83 per cent susceptibility. They were classified as susceptible. Five resistant and five susceptible genotypes from F_2 were selected for bulk segregant analysis.

Genomic DNA for RAPD analysis was isolated by Rogers and Bendich method (1994). Good quality DNA with an absorbance ratio of 1.8-2.0 was used for RAPD analysis.PCR reaction mixtures and conditions for DNA amplification were standardised. Ninty two, 10-12 bp primers belonging to OPA, OPB, OPC, OPF, OPE, OPU, OPH, OPAH, OPAG, OPL, OPM, RY, RN, RA, SC, RF, AG 8, WG 44, GLE11, RF, R10, R6, and PUC101 were initially screened with resistant genotype Surya and susceptible genotype Pusa Purple Long to select primers with polymorphism and good amplification. Out of ninty two primers tested thirty were reported as bacterial wilt specific.

The PCR products were electrophoresed and twenty two primers were selected for BSA based on amplification power and polymorphism. They were RY 01, RY 02, RY 03, RN 19, OPF 5, OPL 04, OPA 04, OPA 6, OPA 9, OPA 24, OPA 26, OPA 34, OPA 36, OPS 9, OPS 10, OPS 16, OPS 17, PUC 101, RA 12-41, and RF. Among these only the primer RY 02 recorded polymorphism between resistant and susceptible variety with an amplicon of 1.2kb.

In bulk segregant analysis DNA of Surya, Swetha, Pusa Purple Long and bulk DNA from resistant genotypes and susceptible genotypes were amplified with selected primers and products were electrophoresed. All primers produced only monomorphic band. None has produced unique polymorphic bands capable of differentiating resistant and susceptible genotypes. This may be due to low polymorphism at genomic level.