TAGGING OF BACTERIAL WILT RESISTANCE GENE IN *Solanum melongena* var. *insanum* BY MOLECULAR MARKERS

By

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(2009-11-104)

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THESIS

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2012

DECLARATION

I, hereby declare that this thesis entitled **"Tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* by molecular markers"** is a bonafide record of research work done by me during the course of research and this thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"Tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* by molecular markers"** is a record of research work done independently by **Mr. Chavan Pradeep Uttamrao (2009-11-104)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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Chavan Pardeep Uttamrao

Dedicated to my beloved Mother and Society

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ABBREVATIONS

%	Percentage
β	Beta
µg	Microgram
µl	Microlitre
µM	Micromole
µg	Micro gram
^{0}C	Degree Celsius
A	Adenine
AFLP	Amplified Fragment Length Polymorphism
AR	Analytical reagent
Avr	avirulence
AVRDC	Asian Vegetable Research and Development Centre
BC	blue colony
BLAST	Basic Local Alignment Search Tool
Blastn	Nucleotide- nucleotide BLAST
Blastp	Protein- protein BLAST
bp	Base pair
BSA	Bulked Segregant Analysis
BW	bacterial wilt
C	Cytosine
$CaCl_2$	Calcium chloride
CAPs	Cleaved Amplified Polymorphic Sequences
cfu	Colony Forming Unit
cM	Centi Morgan
cm	Centimeter
COH	College of Horticulture
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
cv.	cultiver
DH	double haploid

DI	**disease index**
DIC	**Distributed Information Centre**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
dNTPs	**Deoxyribo Nucleoside Triphosphate**
E. coli	***Escherichia coli***
EDTA	**Ethylene diamine tetra acetic acid**
et al.	**et alii (and others)**
EtBr	**Ethidium bromide**
FYM	**farmyard manure**
G	**Guanine**
g	**gram**
HPR	**host plant resistance**
hr	**hours**
HR	**Hypersensitive response**
HSP	**Heat Shock Protein**
i. c. no.	**National identification number**
IARI	**Indian Agricultural Research Institute**
Inc.	**Incorporated**
IPTG	**Isopropylthio-β-D-galactoside**
ISSR	**Inter Simple Sequence Repeat**
JA	**Jasmonic acid**
KAU	**Kerala Agricultural university**
kb	**kilobase**
kDa	**kilo Dalton**
kg	**Kilogram**
L	**Litre**
LB	**Luria Bertani**
LBA	**Luria Bertani Agar**
m	**Meter**
M	**marker**
M	**molar**
M	**mole**
mA	**Milli Ampere**

MAS	**Marker assisted selection**
Mb	**Mega base pairs**
MDa	**Mega Dalton**
mg	**Milligram**
$MgCl_2$	**magnesium chloride**
MilliQ water	**deionized water purified in MilliQ system**
min	**minute**
ml	**Milli litre**
mm	**Millimeter**
mM	**Millimolar**
mM	**milimolar**
MR	**Moderately resistant**
Ms	**Moderately susceptible**
NaCl	**Sodium chloride**
NaOH	**Sodium hydrixide**
NBPGR	**National Bureau of Plant Genetic Resources**
NCBI	**National Centre for Biotechnology Information**
ng	**Nanogram**
NH4OAc	**Ammonium acetate**
NIL	**Near isogenic line**
nm	**Nanometer**
nM	**Nano molar**
no.	**Number**
O.D.	**Optical density**
ORF	**Open Reading Frame**
PCR	**Polymerase Chain Reaction**
pH	**Hydrogen ion concentration**
pM	**picomole**
ppm	**Parts per million**
PR	**Pathogenesis related protein**
pUC	**Plamid of Univesity of Colfornia**
PVP	**Poly vinyl pyrolidine**
QTL	**quantitative trait loci**
R	**Resistance**

R. solanacearum	***Ralstonia solanacearum***
RAPD	**Random Amplified Polymorphic DNA**
RFLP	**Restriction Fragment Length Polymorphism**
RILs	**Recombinant inbreed lines**
RNA	**Ribo Nucleic acid**
RNase	**Ribonuclease**
RP	**Resistant Parent**
rpm	**Rotations per minute**
S	**Susceptible**
S.	***Solanum***
SA	**Salicylic acid**
SAR	**Systemic acquired resistance**
SCAR	**Sequence Characterized Amplified Region**
sec	**Second (s)**
SP	**Susceptible parent**
SSLP	**Simple sequence length polymorphism"**
SSR	**Simple Sequence Repeat**
STS	**Sequence Tagged sites**
T	**Thymine**
TAE	**Tris Acetate EDTA buffer**
TE	**Tris EDTA buffer**
Tm	**Melting temperature**
U	**Unit**
USA	**United States**
UV	**Ultra violet**
V	**Volts**
v/v	**volume/volume**
var.	**variety**
W	**white colony**
w/v	**Weight by volume**
xg	**gravity**
X-gal	**5bromo-4-chloro-3-indoyl- β -D-galactosidase**
µg	**micro gram**
µl	**micro litre**

Introduction

1. INTRODUCTION

Bacterial wilt caused by the soil-born plant pathogen *Ralstonia solanacearum* (Smith, 1896; Yabuuchi *et al.*, 1995) is one of the most devastating bacterial plant diseases in the tropical and subtropical regions of the world. It gained importance in the world due to its destructive nature, wide host range and geographical distribution. It affects a wide range of economically important crops such as tomato, potato, eggplant, chilli and non-solanaceous crops such as banana and groundnut. Vanitha *et al.*, 2009). Yield loss in susceptible varieties varies from 30 per cent under mild infection to 100 per cent under severe infection. The conventional control measures by chemicals and sanitation are not effective in controlling the disease due to its broad host range and soil borne nature. The most widely accepted and promising strategy is breeding resistant cultivars and success stories have been reported in chilli, tomato and eggplant.

Induced protection of plants against various pathogens has been reported since 1930s when Chester (1933) proposed the term acquired physiological immunity. Since then several terms have been used to describe the phenomenon of induced resistance such as systemic acquired resistance (Ross, 1961), translocated resistance (Hurbert and Helton, 1967) and plant immunization (Tuzun and Kuc, 1991). Plants have several inducible defense mechanisms to limit pathogen infection, which include increased lignifications and cell wall cross-linking, production of small antibiotic molecules (i.e. phytoalexins), host cell death at the site of infection i.e. the hypersensitive response; (Bowles, 1990.), production of reactive oxygen species (Mehdy, 1994) and expression of a large set of genes termed pathogenesis-related (PR) genes (Van Loon *et al.*, 1987 and Ward *et al.*, 1991) or R genes (Martin, 1999 and Ellis, *et al.*, 2000).

Defense in plants against pathogen mediated by Resistance (*R*) genes is very much effective in controlling disease and they are able to detect specific pathogen races through recognition of pathogen-encoded Avirulence (Avr) proteins. Resistance mechanism by R genes can be monogenic or polygenic,

dominant or recessive. Gopimony (1983) reported Solanum *melongena* var. *insanum* (wild variety) as resistant to bacterial wilt and the controlling gene is monogenic and dominant.

Biotechnology offers tools for tagging, characterization and isolation of R genes. This can be done with molecular markers coupled with suitable breeding method. Some of the markers used for these purposes include RAPD, AFLP, SSR and ISSR.

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.

Tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* through molecular markers may help in isolation of the gene, which is monogenic and dominant. Development of molecular marker linked to bacterial wilt resistance in *Solanum melongena* var. *insanum* will help in marker assisted selection, while transferring this character to genetic background of other cultivated crops for imparting bacterial wilt resistance.

The present study involves molecular characterization of resistant and susceptible genotypes using PCR based RAPD and STS markers for tagging bacterial wilt resistant gene. Resistant variety *Solanum melongena* var. *insanum*, susceptible variety Pusa Purple Long and segregating F_2 population of cross *Solanum melongena* var. *insanum* x Pusa Purple Long served as source of DNA for molecular marker analysis.

The study "Tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* by molecular markers" was undertaken to identify a molecular marker linked to bacterial wilt resistance in *Solanum melongena* var. *insanum*.

Review of literature

2. REVIEW OF LITERATURE

The research programme "Tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* by molecular markers" was selected for developing a molecular marker linked to bacterial wilt resistance in *insanum* a wild variety of brinjal. The relevant literature on various aspects of the investigation is reviewed in this chapter.

2.1. Bacterial Wilt disease

Bacterial wilt caused by the soil-borne plant pathogen *Ralstonia solanacearum* (Smith, 1896) is one of the most devastating bacterial plant diseases of the tropical and subtropical regions of the world. It gained importance in the world due to its destructive nature, wide host range and geographical distribution. It affects a wide range of economically important crops such as tomato, potato, eggplant, chilli and non-solanaceous crops such as banana and groundnut (Vanitha *et al*., 2009).

Bacterial wilt caused by *Ralstonia solanacearum* was first reported at the end of the 19th century on potato, tobacco, tomato and groundnut in Asia, southern USA and South America. The bacterium was described for the first time as *Pseudomonas solanacearum* by Smith (1896). This species was known for many years as *Pseudomonas solanacearum* E. F. Smith. The new genus *Ralstonia* was established to accommodate *R. solanacearum* together with the closely related species within the rRNA homology group II, *R. picketii* and *R. eutropha (*Yabuuchi *et al*., 1995).

Ralstonia solanacearum (Smith 1896, Yabuuchi *et al*., 1995), causal agent of bacterial wilt, is an important Gram-negative plant pathogenic bacterium in tropical and subtropical areas, infecting over 200 plant species belonging to more than 50 botanical families (Hayward, 2000). This bacterium is highly heterogeneous in nature, and the pathogen has been divided into five races

according to host ranges and six biovars by the ability to utilize sugar alcohols and disaccharides (Hayward 1964; He *et al.*, 1983; Hayward *et al.*, 1990). In the years following, at least five pathogenic races and five biovars have been discriminated (Buddenhagen *et al.*, 1962). Race 1 occurs in tropical areas all over the world and attacks tobacco, many other solanaceous crops and has many hosts in other plant families. It has a high temperature optimum (35 °C, as do race 2, 4 and 5). Race 2 occurs mainly in tropical areas of South America and attacks bananas and *Heliconia* (causing so Moko disease) and are also seen in Philippines (causing bugtok disease on plantains). Race 3, occurring at higher altitudes in the tropics, subtropical and temperate areas attacks potato, tomato, and occasionally *Pelargonium zonale*, aubergine, capsicum, some solanaceous weeds like *Solanum nigrum* and *Solanum dulcamara*. A number of non-solanaceous weed hosts have also been found to harbour race 3 infections, often asymptomatically (Pradhanang *et al.*, 2000; Strider *et al.*, 1981; Wenneker *et al.*, 1999; Janse *et al.*, 2004). This race has a lower temperature optimum (27°C) and appears to be mostly biovar 2.

Recently, Fegan and Prior (2005) and Murugaiyan *et al.*, 2010 proposed a new hierarchical classification of *R. solanacearum* into four phylotypes based on phenotypic and molecular typing that are highly consistent and provide more insight into the pathogenicity and geographical origin of the strains . This will contribute to better understanding of the interactions between the resistance and the bacterial strains used in breeding programs (Fegan and Prior, 2005). This may be useful to explore bacterial wilt resistant markers in resistant sources.

2.2. Symptomatology of the disease

Bacterial wilt of solanaceous vegetables appears as a sudden wilt of foliage. The first visible symptom is wilting of the leaves at the ends of the branches during the heat hours of the day with recovery at night. Eventually, plants fail to recover and die rapidly within 3-4 days. As the disease develops, a streaky brown discoloration of the stem may be observed on stems up to 2.5 cm or more above the soil line, and the leaves have a bronze tint. Moreover epinasty of the petioles

may occur. Bacterial masses prevent water flow from the roots to the leaves, resulting in plant wilting. Severity of the disease depends on soil temperature, soil moisture, soil type (which influences soil moisture and microbial populations), host susceptibility, and virulence of strains. High temperature (30-35^{o}C) and high soil moisture are the main factors associated with high bacterial wilt incidence and severity. Under these conditions, high populations of bacteria are released into the soil from the roots as the plant wilts. The disease can be easily detected by ooze test of small pieces of wilted plant stem within clear water (Ghosh and Mandal 2009). This test has presumptive diagnostic value in the field.

2.3. Methods of screening

Several artificial inoculation methods are effective in distinguishing reaction of genotypes with different resistance levels to bacterial wilt.

Winstead and Kelman (1952) evaluated the relative effectiveness of various procedures to inoculate susceptible and resistant tomato plants. Preliminary tests with naturally infested soils showed the superiority of pure cultures for inoculation under green house conditions. They aimed a procedure that would ensure uniform and rapid development of bacterial wilt. Pathogenecity test of bacterial cultures by inoculating tomato plants by stem puncture method lead to rapid wilting and at the end of 14 days, all inoculated plants were wilted.

Wang (1971) reported artificial inoculation in tomato by leaf clipping method was effective for delivery of pathogen into plant system. Lin *et al.* (1974) inoculated tomato plants by clipping off leaf tips with scissors dipped in a suspension of bacterial culture and obtained wilting to the same extend as found in plants grown in naturally infested field. Sheela (1982) reported that dipping of seedlings in fresh bacterial ooze just before transplanting followed by inoculation of seedlings in the leaf axils resulted in 100 per cent infection in susceptible brinjal seedlings.

Kishun and Chand (1988) conducted an experiment with seven inoculation methods by using 25 days old susceptible tomato cv. Pusa Ruby in nutrient solution. They found that the system can be successfully used for evaluation of virulence of *Ralstonia solanacearum* and the best method was leaf axil inoculation. Butranu *et al.* (1994) tested several inoculation techniques and found petiole clipping method as the best for resistance screening.

2.4. Mechanism of wilting

Entry of *Ralstonia solanacearum* into plants is by way of injured roots, stem wounds or through stomata. Within the plant, the bacteria move in the vascular bundles, a process which is accelerated by higher temperature. Speed of movement is also dependent on the plant part colonized, for instance in tobacco bacteria move quicker in the stalk than in the roots (Ono *et al.*, 1984). This is followed by colonization of the xylem (Xiao *et al.*, 1983) and then progress into the intercellular spaces of cortex and pith causing lysigenous cavities. Break down of plant tissues due to bacterial wilt is attributed to the cellulose and poly-galacturonase enzymes produced by the bacterium (Hussain and Kelman, 1957). The bacteria adhere by polar attraction to the cell surfaces and subsequently become localized at preferential sites of the mesophyll (Petrolini *et al.*, 1986). Blocking of the vessels by bacteria is the major cause of wilting.

Severe wilting is caused by vascular plugging even when inoculated with weakly virulent strains. All the virulent strains produce an extra cellular slime, a wilt inducing material (Hussain and Kelman, 1957). They suggested that the virulent strains after entry into the host multiply rapidly in xylem and form slime in abundance which causes a marked increase in the viscosity of vascular stream and interfere with water movement resulting in wilting. Bacterial colonization of the stem results in browning of the xylem, foliar epinasty and lethal generalized wilting (Buddenhagen *et al.*, 1962).

2.5. Genetic nature of bacterial wilt resistance

Resistance sources have been identified in some wild related of species tomato and brinjal, such as *Solanum torvum* and *Solanum aethiopicum* (Gousset *et al*., 2005), and their resistance is greatly affected by environmental factors and the race and strain diversity of the pathogen, which makes it very difficult to utilize these resistance sources in different countries.

Tomato

Studies on inheritance of resistance to BW caused by *R. solanacearum* in tomato are complex and different results have been obtained using different materials. Various degrees of varietal resistance have been reported. The resistant genes had been defined as recessive (Singh, 1961; Acosta *et al*., 1964; Mohamed *et al*., 1997), incomplete dominance (Graham and Yap 1976; Yue *et al*., 1995; Mohamed *et al*., 1997; Shou *et al*., 2006) and dominance (Scott *et al*., 1988; Grimault *et al*., 1995). Many resistant genes had been defined as monogenic (Scott *et al*., 1988; Gowha and Shivasbankara, 1990; Grimault *et al*., 1995; Li *et al*., 2001) or oligogenic (Yue *et al*., 1995, Gilbert *et al*., 1974, Osiru *et al*., 2001), or polygenic (Acosta *et al*., 1964; Gilbert *et al*., 1974; Thurston, 1976; Gonzalez and Summers, 1995; Yue *et al*., 1995; Osiru *et al*., 2001). Some researchers reported these genes had additive (Graham and Yap, 1976; Yue *et al*., 1995; Hanson *et al*., 1998; Balatero *et al*., 2000) and non-additive effect (Mohamed *et al*., 1997; Balatero *et al*., 2000) between genes.

Breeders at AVRDC generally have treated bacterial wilt resistance as a polygenic trait despite the conflicting theories on bacterial wilt genetic system (Acosta *et al*., 1964; Bosch *et al*., 1985; Singh, 1961; Tikoo *et al*., 1990). Bacterial wilt resistant stocks North Caroline, for which polygenic inheritance has been implicated have been frequently used as resistant parent (Opena and Tschanz *et al*., 1987). Moreover, it is also doubtful that the major bacterial wilt resistance genes, such as those reported (Bosch *et al*., 1985, Tikoo *et al*., 1990) would be

stable over the wide geographical areas that have to be encompassed by AVRDC's advanced tomato lines (Opena *et al.*, 1987, 1990).

Many BW resistant loci and molecular markers have been found in tomato plants. Using F_2 and F_3 population derived from 'L285' (resistant) × 'CLN286' (susceptible), Danesh and Young, (1994) found 3 QTLs. Thoquet *et al.* (1996) found 7 QTLs using the F_2 and F_3 population derived from *Lycopersicon esculentum* cv. 'Hawaii 7996' (partially resistant) × *Lycopersicon pimpinellifolium* 'Wva700' (susceptible), while Carmeille *et al.* (2006) found 4 QTLs with a $F_{2:3}$ and a population of inbred lines (RIL). Balatero *et al.* (2002) found 80 molecular markers, including 72 AFLP markers, 7 resistance gene analogs, 1 SSR marker, during the construction of a tomato linkage map of 12 linkage groups. Yui *et al.* (1999) developed four RAPD markers which were useful for preliminary selection of bacterial wilt resistance, introduced by resistant parent 'Hawaii 7998'. Two markers, namely RA-12-13_{450} and RA-12-$29_{1600,}$ were found to be linked to a resistance gene with a large effect. Most of the loci and markers are on the chromosome 6 and other chromosomes such as chromosome 4, 7, 8, 10, 11 and 12 also contribute to the control of the BW resistance (Wang *et al.*, 2000, Thoquet *et al.*, 1996, Mangin *et al.*, 1999).

According to Ferrer 1974; Acosta *et al.*, 1964; and Monma and Sakata 1983, BW resistance is quantitative and there is difficulty for breeding resistant cultivars. Molecular markers and detailed genetic maps based on RFLPs have made great contribution in elucidating the genetic nature of quantitative traits in various crops (Osborn *et al.*, 1987, Paterson *et al.*, 1988, Tankley and Hewitt 1988).

Potato

Wild *Tuberosa* and *Solanum phureja* (Hawkes, 1990) are phytogenetically close to *Solanum tuberosum* and displays resistant traits, dominant and readily transmitted to progeny. Some clones of *S. phureja* with high degree of resistance to bacterial wilt have been used for crossing with commercial cultivars of

S.tuberosum French *et al.*, 1998 and Sequeira and Rowe, 1969. Resistance to bacterial wilt derived from *S. phureja* was first described as dominant and controlled by three unlinked genes (Buddenhagen, 1986; Rowe and Sequeira 1970; and French and De Lindo 1982). More recently, at least four major genes have been reported to be involved in potato resistance to bacterial wilt (Grimley and Hanson 1998, Fock *et al.*, 2000).

Miao *et al.*, 2007 reported many genes or regulators involved in determining resistance to several strains of the causal agent of BW, *R. solanacearum*, such as virulence genes pehR, vsrB, vsrD, rpoS, hrcC, pme and gspK. The reference about these genes come from Schell, 2000, Flavier et al. 1998, Tans-Kersten *et al.*,1998, Kang *et al.*, 1994, Arlat *et al.*, 1992. RRS1 (Deslandes *et al.*, 2002), ipx genes (Darby and Brown, 2004), TSRF1 (Zhang *et al.*, 2007) have also been identified. RRS1-R is a recessive gene and RRS1-S is a dominant gene, conferred resistance to *R. solanacearum* in Arabidopsis. They encode highly similar predicted proteins differing in length and RRS1-R behaves as a dominant resistance gene in transgenic plants.

Eggplant

The inheritance of resistance to BW caused by *R. Solanacearum* in eggplant is complex and different results have been obtained using different materials. Various degrees of varietal resistance have been reported (Messiaen, 1975; Mochizuki and Yamakawa, 1979; Che *et al.*, 1997). Depending on the varieties used, resistance to *R. Solanacearum* is controlled by one dominant gene (Chadha, 1993; Chaudhary, 2000; Zhu *et al.*, 2004; Yang *et al.*, 2006; Ajjappalavara *et al.*, 2008) or one recessive gene (Chaudhary, 2000; Gopalakrishnan *et al.*, 2005; Li *et al.*, 2006; Tian *et al.*, 2007; Sun *et al.*, 2008), or polygene (Chaudhary, 2000; Li *et al.*, 2002) or recessive polygene (Feng *et al.*, 2003). studies of Swaminathan and Srinivasan (1972), Gopinath and Madalageri (1986), Zhu *et al.*, (2004), Yang *et al.* (2006) and Ajjappalavara *et al.* (2008), also suggest that single gene inheritance for bacterial wilt resistance. Gousset *et al.* (2004) found that bacterial

wilt resistance could be interfered by cytoplasmic factors also. Bi-hao *et al.* (2009) reported inheritance of bacterial wilt resistance in eggplant was controlled by a single dominant gene.

Up till now, many molecular markers related to resistant BW have been reported, such as RAPD marker located at 4.33 cM from the monogenic dominant resistance gene (Zhu *et al.*, 2005), two AFLP markers related to monogenic recessive resistance (Li *et al.*, 2006; Sun *et al.*, 2008) or one 400 bp RAPD marker related to polygenic dominant resistance (Li *et al.*, 2002), but the function of these resistant markers were still not identified.

Gopimony (1983) had conducted genetic analysis of bacterial wilt resistance in *Solanum melongena* var. *insanum* (wild variety) by crossing the same with cultivated variety Pusa Purple Giant. He found that F_1 is resistant and susceptible F_2 plants showed resistance at the ratio of 3:1. Genetic analysis showed that the bacterial wilt resistance in this wild brinjal is monogenic and dominant.

Jessykutty (1985) reported that SM 6-2 and SM 6-4 brinjal genotypes were highly resistant after screening under field conditions. Geetha and Peter (1993) 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.

Narayanan (1984) selected three wilt resistant varieties *viz.*, Pusa Purple Cluster, SM 1-10 and SM-6 as female parents 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.

Varma (1995) found that progenies of the crosses SM 6-6 x SM 197, SM 132 x SM 262 and SM 141 x SM 262, genotypes of brinjal 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.

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.

Gokulapalan *et al.* (2004) reported high resistance to the most virulent isolates of *R. solanacearum* in the variety Surya (SM 6-7).

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.

Bi-hao *et al.* (2009) 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). He reported inheritance of bacterial wilt resistance in eggplant was controlled by a single dominant gene.

Gilbert *et al.*, 1974 reported the resistance is controlled by two heterozygous and incomplete dominant genes, and the two genes have some complementary effect. There are mainly two reasons for resistance differences. One is the impersonality factor, including resistant materials, strains of *R. solanacearum*, microbial interaction and second is environment. Similar mechanisms have been postulated to occur in tomato (Hikichi *et al.*, 1999; Nakaho *et al.*, 2000).

2.5.1. Strain and location specificity

Resistance source to bacterial wilt in tomato have been identified and cultivers with different level of resistance have been developed (Scott *et al.*, 1988). However, breeding for durable resistance is challenging due to the fact that resistance in tomato to bacterial wilt can be location-specific (Hanson *et al.*,

1996). Location specificity can be due to the presence of strains that vary in aggressiveness. For example, all reported strain of *R. solanacearum* race 1 and biovar 3 or 4 in Taiwan, are highly variable in aggressiveness (Jaunet and Wang, 1999). Performance of a resistant line is largely depending on local strain profiles. Durability of selected resistance source is a concern, as high temperature and strain can affect the severity of bacterial wilt on tomato. It is known that several tomato varieties displayed higher severity of bacterial wilt under high temperature under controlled environment (Krausz and Thurston, 1975) and in the field (Fegan and Prior, 2005). And strain-specific QTLs have already been identified in H7996, a resistant tomato variety (Danesh and Young, 1994; Wang *et al.*, 2000). Danesh and Young, (1994) demonstrated that the resistance controlled by this locus could be strain specific.

2.6. DNA isolation in solanaceous crops

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).

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.

Karumannil (2007) reported that protocol suggested by Rogers and Bendich (1994) was ideal for DNA isolation from tomato leaves. Li *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.

Mirshamsi *et al.* (2008) isolated genomic DNA from tomato hybrids based on modified Dellaporta 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.

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.

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.

2.7. Molecular characterization

The detection of naturally occurring DNA sequence polymorphisms between individuals and using that as molecular markers for crop improvement represent one of the recent developments in molecular biology. The pace of DNA-based mapping and DNA-based markers selection has been accelerated in the past few years by the advent of the PCR (Saiki *et al.*, 1988; Mullis and Faloona, 1987; Mullis *et al.*, 1986). This facilitated development of marker-based gene tags, map-based cloning of agronomically important genes, variability studies, phytogenetic analysis and marker-assisted selection of desirable genotypes.

Genetic Markers of heritable entities that are associated with economically important traits can be used by plant breeders as selection tools (Darvasi and Soller, 1994) and it is called as Marker Assisted Selection (MAS). Marker assisted selection or marker aided selection is a process whereby a marker (morphological, biochemical or molecular markers) is used for indirect selection of a genetic determinant or determinants of a trait of interest. It is indirect selection process where a trait of interest is selected not based on the trait itself but on a marker linked to it**.** Molecular marker is based on DNA or RNA variation in phenotype.

MAS can be useful for traits that are difficult to measure, exhibit low heritability, and/or are expressed late in development. Morphological, Biochemical and Molecular markers comes under this. Compared to biochemical marker, DNA markers give a much higher degree of polymorphism and stability.

Molecular markers are especially advantageous for tagging agronomic traits and are used to tag traits as resistance to pathogens, insects, and nematodes, tolerance to abiotic stresses, quality parameters and quantitative traits. Molecular marker studies using near isogenic lines (Martin *et al*., 1991), recombinant inbreed lines (Mohan *et al*., 1994) or bulked segregant analysis (Michelmore *et al*., 1991) has accelerated the mapping of many genes in different plant species. PCR based molecular markers (e.g. RAPD, SCAR, CAPs, STS and SSLP) are preferred over hybridization based markers like RFLP. Among the PCR based markers, RAPD and Microsatellite have been exploited in genome mapping, DNA fingerprinting, genetic diversity analysis and gene tagging (Gupta and Varshney, 2000; Xiao *et al*., 1996).

Restriction fragment length polymorphism (RFLP) analysis is a powerful tool for developing precise high-density molecular genetic maps because it reveals reliable and stable polymorphism. Wongse *et al*. (1994) reported tagging of a powdery mildew resistance gene in tomato using RFLP markers. Markers based upon DNA probes have introduced a new dimension to the development of genetic maps and the mapping of agronomically and physiologically important characters. (Wyman and White, 1980).

Microsatellites have proved to be potentially useful markers for genome mapping and DNA fingerprinting (Yu *et al*., 1999). Simple sequence repeat polymorphism has been demonstrated to be a powerful tool in gene tagging and QTL analysis (Blair and McCouch, 1997; Xiao *et al*., 1996), pedigree analysis and marker-assisted breeding (Ayres *et al*. 1997).

2.7.1. Molecular markers in mapping disease resistance genes in crops

Molecular markers have opened up new opportunities for studies in host plant resistance (HPR). They hold good promise to trace genes conferring resistance to pathogens. Molecular genetic maps are now available for solanaceous crops viz, tomato and other field crops like rice. If the genes for pathogen resistance can be tagged by molecular markers with tight linkage, selection efficiency can be increased and the time and money in moving these genes from one varietal background to another can be greatly reduced. Molecular marker technique has made it feasible to map the major genes, polygene and candidate genes associated with resistance to bacterial wilt (Yagi *et al*., 2006).

Many of the identified major genes for bacterial wilt resistance have been mapped to specific chromosomal locations of genome using the molecular marker technique. The strategy followed to tag major genes includes parental and progeny survey, followed by construction of near isogenic line (NIL). Since construction of NIL is time consuming and tedious, major genes can be mapped by another alternative strategy, bulked segregant analysis (Michelmore *et al*., 1991).

Jena *et al*. (1992) reported BPH resistance gene has been introduced into cultivated rice lines from a distantly related species of *Oryza* and the gene has been mapped with a DNA marker by RAPD and bulked segregant analysis method.

Zhu *et al*. (1993) mapped blast resistance gene, *Pikh* in rice with RAPD marker using double haploid lines, and the linked RAPD marker was later mapped to rice chromosome 8.

Stevens *et al*. (1995) mapped *SW-5* which confers resistance to tomato spotted wilt virus (TSMV) in tomato and acts as a dominant gene between two RFLP markers (CT 27 and CT 220) near telomeric area of chromosome 9, and also identified one RAPD marker that is localized within 0.5 cM of *SW-5*. Chague *et al*. (1997) identified six RAPD markers linked to the *SW-5* gene using bulked

segregant analysis. One of the coupling markers close to the gene was used to develop a SCAR marker.

2.7.2. Tagging of disease resistant genes with molecular markers

The process of locating genes of interest via linkage of markers is referred as "gene tagging" (Chawla, 2002). RAPD, RFLP, SSR, SCAR and STS markers are suitable for tagging major gene controlling disease resistance, pest resistance etc. In rice, gene conferring resistance to blast was tagged using RAPD markers (Zhu *et al*., 1993).

2.7.2.1. RAPD for tagging resistance genes

The RAPD technique is based on the use of single arbitrary oligonucleotides as primers for PCR (Williams *et al*., 1990). These primers are synthetically produced random DNA sequences, approximately 10 nucleotides in length, with 50-60 per cent GC content. RAPD markers have been proposed as an alternative to RFLP (Williams *et al*., 1990) and require less DNA, do not require southern blotting and radioactive labeling, and are relatively quick to assay. It is inherited in a Mendelian fashion and can be generated without any prior knowledge of the target DNA sequence (Welsh *et al*., 1991). It is inherited as dominant markers, where the presence of a particular band is dominant, and its absence is recessive (Tingley and Tufo, 1993). RAPD analysis has found applications in population studies (Welsh *et al*., 1991), biosystematics (Stiles *et al*., 1993), gene tagging (Naqvi and Chattoo, 1995) and fingerprinting (Mackill, 1996; Virk *et al*., 1995).

Martin *et al*. (1991) surveyed polymorphism for bacterial wilt against *Ralstonia solanacearum* in the near isogenic line (NILs) of tomato through RAPD and identified three markers. These markers were identified to be linked to '*Pto*' gene conferring resistance to the bacterial wilt.

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.

Michelmore (1995) provided new opportunities for accessing the great diversity of disease resistance genes in various crop plants by RAPD marker. Zhang *et al.*, (1997) identified six RAPD markers associated with the nuclear fertility-restoring gene *Rf-3*. Three of these OPK 5_{800}, OPV 10_{1100} and OPW 1_{350} were mapped on chromosome 1.

RAPD analysis was conducted with the combined use of near isogenic lines and BSA to tag the bacterial blight resistance genes *Xa*-21, *Xa*-3, *Xa*-4 and *xa*-5 in rice (Yoshimura *et al.*, 1995; Zhang *et al.*, 1994). From the survey with 260 decamer primers, OPAC 5 was detected to amplify specifically a 0.9 Kb fragments from the DNA of bacterial blight susceptible plants. The distance between the RAPD marker OPAC 5_{900} and *Xa*-13 was estimated to be 5.3 cM (Zhang *et al.*, 1996).

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.

Caranta *et al.* (1997) reported the tagging of a powdery mildew *Leveillula taurica* (Lev.) Arnaud resistance gene (*Lv*) in tomato using RAPD and RFLP markers. DNA from a resistant and a susceptible cultivars were screened with 300 random primers and four primers yielded fragments that were unique to the resistant line and also linked to the resistance gene in F_2 population. One of these amplified fragments with OP248, with a molecular weight of 0.7 Kb, was subsequently mapped to chromosome 12.

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.

Shen *et al.* 1998 surveyed two F_2 populations and one BC_1 population to confirm the linkage of the markers for fertility restorer gene *Rf*-3 gene and they tagged the same with RAPD marker OPB 18_{1000} at a distance of 5.3 cM in chromosome 1.

Yi *et al.* (1998) reported that the root-knot nematode resistance gene *Rk* which confers resistance to races 1 and 3 of the root-knot nematode (*Meleoidogyne incognita*) has been mapped in tobacco with RAPD markers and they used two varieties NC528, KY14 and three Rk-resistant DNA bulks, and three Rk-susceptible bulks generated from F_1-derived maternal doubled haploid (MDH) individuals for bulked segregant analysis.

Jeon *et al.* (1999) used BSA and tagged RAPD marker OPD 7 to the rice *bph 1* gene conferring resistance to biotype 1 of BPH. OPD 7 yielded a 700 bp fragment which was present in resistant parent and resistant F_2 plants but absent in susceptible parent and susceptible F_2 plants. Chromosomal regions surrounding the *Bph-1* were examined with additional RFLP and microsatellite markers on chromosome 12 to define the location of the RAPD marker and *Bph-1*.

Sandhu *et al.* (2002) carried out RAPD analysis of herbicide-resistant Brazilian rice lines produced via mutagenesis. Among eighty random primers tested, 10 were selected for a detailed study of RAPD markers that could tag herbicide resistance genes. Resistant and susceptible lines produced variation in the RAPD patterns and certain bands were found only in certain lines. Twenty lines resistant to herbicide were selected as a result of this study.

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.

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.

Nematzadeh *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.

Yagi *et al.* (2006) constructed genetic linkage map in carnation for bacterial wilt resistance on the basis of 137 RAPD primers and 9 SSR primers which showed polymorphism in resistant line 'Carnation Nau No.1' and which lead to mapping of quantitative trait loci (QTL)

Divakaran (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.

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.

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.

2.7.2.2. STS for tagging resistance genes

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 (60 to 1000 bp) that can be amplified by PCR, which identifies a known location on a chromosome (Olson *et al*., 1989). The Sequence-Tagged Site (STS) is a relatively short, codominant, reproducible, easily PCR-amplified sequence (200 to 1000 bp) which can be specifically amplified by PCR and detected in the presence of all other genomic sequences and whose location in the genome is mapped. It can distinguish between homozygotes and hetrozygotes. It need some pre-existing knowledge of the DNA sequence of region.

Examples of STSs are Sequence tagged microsatellite (STMs), Sequence characterized amplified regions (SCARs), Cleaved amplified polymorphic sequence (CAPs)

Fakuoka *et al*. (1992) 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.

Mohan *et al.* (1994) and Nair *et al.* (1995) used BSA and tagged RAPD markers OPF 8 and OPF 10 to the rice *Gm2* gene that confers resistance to biotype 1 of gall midge in rice. The *Gm4t* gene for gall midge resistance was also tagged by RAPD markers E 20_{570} and E 20_{583} which were then converted into sequenced tagged sites.

Wongse *et al.* (1994) reported the tagging of a powdery mildew resistance gene in tomato using RFLP markers.

Bulked segregant analysis on F_2 population of Nipponbare x Kasalath cross was used to determine RAPD markers in a specific interval in the middle of the chromosome 6 of rice for tagging the photoperiod sensitivity gene. Fourteen markers tightly linked to the photoperiod sensitivity gene were identified. They were converted into STS by cloning and sequencing the polymorphic fragments (Monna *et al.*, 1995).

Hittalmani *et al.* (1995) developed a PCR-based marker using RFLP marker RG64, which is tightly linked to blast resistant gene *Pi-2* and generated three PCR products known as sequence tagged sites (STS). The amplified products were cleaved with restriction enzymes *HaeII* and produced specific amplicon.

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.

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.

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.

Onozaki *et al.* (2004) reported a RAPD-derived STS marker linked to a bacterial wilt (*Burkholderia caryophylli*) resistant allelic gene in carnation. A locus with large effect on bacterial wilt resistance was mapped around WG44-1050 through QTL analysis. Then WG44-1050 was converted to a STS marker for marker assisted selection.

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.

2.7.2.3. SCAR for tagging resistance genes

A SCAR is a genomic DNA fragment at a single genetically cloned locus that is identified by PCR amplification using a pair of specific oligonucleotide

primers (Williams *et al.*, 1991). Frequently mispriming error amounted in replication studies due to frequently observed problems with reproducibility of RAPD profiles and specific bands. To overcome this problem, Paran and Michelmore (1993) converted RAPD fragments to simple and robust PCR markers, termed sequenced characterized amplified region (SCAR). It 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).

Naqvi and Chattoo (1995) reported the identification of two RAPD markers OPF 6_{2700} and OPH 18_{2400} linked to *Pi-10* blast resistance gene in rice. These markers were converted into SCARs and based on the polymorphisms appearing as differences in the length of the SCARs alternate were considered for the indirect selection of *Pi-1*

Chunwongse *et al.* (1997) developed high resolution genetic map in tomato and tagged *Lv* resistance locus, which confers powdery mildew resistance by RAPD marker. Tai *et al.* (1999) studied expression of the *Bs2* pepper gene which confers resistance to bacterial spot disease in tomato.

Procunier *et al.* (1997) tagged the R-gene T_{10} with SCAR marker linked to loose smut resistance gene which caused by *Ustilago Segatium tritici* in wheat.

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.

Kasai *et al.* (2000) developed SCARs based on nucleotide differences within resistance gene like fragments isolated from a potato plant carrying the *Ryadg* gene, which confers extreme resistance to potato Y potyvirus (PVY). It originates from *Solanum tuberosum* subsp. *andigena*, and a susceptible potato

plant. The SCAR marker RYSC3 was generated only in genotypes carrying *Ryadg* gene.

Furini and Wunder (2004) reported molecular analysis of 94 Solanum accessions, including eggplants and related species using AFLP technique. Moon Nicholson (2007) 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.

Potato virus Y (PVY) is the only potyvirus infecting *Capsicum annuum* L., a monogenic dominant gene *Pvr4* confers resistance to PVY pathogen. Andres *et al.* (2004) conducted BSA to search for RAPD markers linked to the *Pvr4* gene using segregating progenies obtained by crossing a homozygous resistant 'Serrano Criollo de Morelos-334' with a homozygous susceptible 'Yolo Wonder' cultivar. Eight hundred decamer primers were screened to identify one RAPD marker UBC 19_{1432} linked in repulsion phase to *Pvr4*. This marker was converted into a dominant SCAR marker SCUBC 19_{1423}.

Sugita *et al.* (2004) converted two RAPD marker E18286 and E18272 into SCAR marker. A PCR analysis using DH (double haploid n=176) and backcross (n=190) population revealed that all the SCAR markers PMFR11269, PMFR11283 and PMFR21200, co-segregated with original RAPD marker. It was mapped at a distance 4.0 cM from L^3 gene responsible for bacterial wilt and blight resistance in *Capsicum*.

Quirin *et al.* (2005) reported SCAR primers for the detection of *phyto.5.2* a major QTL for resistance to *Phytophthora capsici* Leon. in pepper. They screened genotypes of *C. annuum* and *C. chinese* with a series of RAPD primers. One primer, OpDO4, amplified a single band only in those *C. annuum* and *C. chinense*

genotypes showing the highest level of resistance and amplified product was cloned, sequenced and converted to a SCAR marker. These primers were observed to define locus on *Capsicum* chromosome 5 which was tightly linked to *phyto.5.2*.

Julio *et al*. (2006) reported SCAR markers linked to three disease resistances viz., black root rot, blue-mold and potato virus Y (PVY) were reported in a *Nocotiana tabaccum* L. accession by AFLP assay.

Tomato spotted wilt virus (TSMV) is a serious disease in tobacco (*Nicotiana tabaccum* L.). The breeding line "Polalta" contains a single dominant gene conferring resistance to TSMV that was introgressed from *N. alata* Link and Otto. The DNA bulks from susceptible and resistant doubled haploid lines derived from a cross between susceptible cultivar 'K326' and "Polalta" were analysed to identify resistance gene by AFLP technology and bulked segregant analysis. Four AFLP fragment were successfully converted to SCAR marker *(*Moon and Nicholson, 2007).

Singh *et al*. (2006) 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.

Techawongstein *et al*. (2006) studied the inheritance of pungency in *Capsicum* by SCAR marker and they crossed the highly pungent variety "Yodson" (YS), local commercial variety of Thailand and the low pungent variety. "YuYi" (YY) from China. They conducted bulked Segregant analysis and reported capsaicinoid content broad sense heritability (h_b^2) and DNA finger print with UBC 20 SCAR primer. They compared results among F_2 populations and

their parents. The SCAR primer for UBC 20 amplified a common fragment of 1700 bp in both low and high pungency chilli plants of all generation.

Miao *et al.* (2007) reported PCR based AFLP marker linked to the genes that confer resistance to tomato bacterial wilt. Bulked segregent analysis of F_2 population showed that tomato bacterial wilt was conferred by two incomplete dominant genes. They screened 256 primers, out of which two AFLP markers were linked to bacterial wilt resistance in tomato. The AFLP markers were converted into a co-dominant sequence characterized amplified region (SCAR) marker named $TSCAR_{AAT/GGA}$ and $TSCAR_{AAG/CAT}$.

Kim *et al.* (2008) developed molecular marker linked to the L4 locus conferring resistance to tobamovirus pathotype in pepper plants, they performed AFLP with 512 primer combinations for susceptible 'S pool' and Resistant 'R pool'. A total of 19 primer pairs produced scorable bands in the R pool. Further screening with these primers pairs was done on DNA bulks from T102, a $BC_{10}F_2$ derived from T10 by back crossing. Each bulk was made by pooling of DNA of five homozygous individuals from a T10 population, which was a near-isogenic BC_4F_2 generation for the L4 locus. AFLP markers were finally selected and designated L4-a, L4-b and L4-c, whereas no recombination for L4-b was seen in 20 individuals of each DNA bulk. L4-b was successfully converted into a simple 340 bp SCAR marker designated L4SC340, which mapped 1.8 cM from the L4 locus in T102 and 0.9 cM in another $BC_{10}F_2$ population.

Rashmi 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.

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.

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.

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. SCAR primers were designed from the sequence which was able to distinguish female gender.

2.8. 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 selected polymorphic 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. BSA removes the need for screening the

entire mapping population with every primer. It 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.

Wang *et al.* (1995) used RAPD markers and BSA to identify molecular markers linked to *Pi-ta* gene in rice which resides on chromosome 12 and flanking between SP4B9 and SP9F3 RAPD markers. Three RAPD markers OPK 17_{1400}, OPA 7_{550} and OPB 10_{450} were reported to be co-segregated with resistance phenotype of neck blast in Gumei 2. Resistance gene was located between OPK 17_{1400} and OPA 7_{550} having genetic distance of 2.4 cM to OPK 17_{1400} and 7.5 cM to OPA 7_{550}.

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 and 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.

Nirmal-Jyothi *et al.* (2001) used RAPD makers and BSA to identify molecular markers linked to *Magnaporthe grisea* Cav. disease resistance in rice.

RAPD analysis and BSA were followed to identify four phenotype specific markers for yellow stem borer resistance in rice. The markers CI_{320} and K_{695} were linked with resistance phenotype whereas markers AH 5_{660} and C 4_{1300} were linked with susceptible phenotype.

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 bacterial 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 $F_{2\text{-}3}$ 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.

Milla *et al.* (2005) identified RAPD and SCAR markers linked to the blue mold resistant tobacco cultivars which is caused by fungal pathogen *Peronospora tabacina*. BSA was used to screen for polymorphisms between DNA bulk from susceptible and resistant cultivar using 1216 RAPD primers. Fifteen RAPD markers were identified as being linked to the major resistance locus to blue mold and two RAPD markers flanking the most likely QTL position were converted to SCAR markers.

Minamiyama *et al.* (2005) studied the character of pungency in pepper (*Capsicum annuum* L.) which is controlled by a single recessive gene (c) and

developed a molecular marker linked to the c-locus using two segregating F_2 population (TM2 and TF2) derived from crosses between pungent and non-pungent peppers in *C. annuum*. Using RAPD technique in combination with bulked segregant analysis, two RAPD markers, OPD 20_{800} and OPY 9_{800} were obtained.

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

Moon and Nicholson (2007) studied the resistance source in tobacco for tomato spotted wilt virus (TSMV) in the breeding line 'Polalta', which contains a TSMV resistance gene introgressed from the wild relative *Nicotiana alata*. He applied AFLP technology and BSA to identify markers linked to TSMV resistance. Primer combinations of 128 numbers were used to screen one resistant and two susceptible bulks and found 48 potential markers linked to the TSMV resistance out of which four AFLP fragments were converted to SCAR markers.

Materials and Methods

3.MATERIALS AND METHODS

The study on 'Tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* by molecular markers' was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period from 2009 to 2011. The plant materials used and methodology adopted in the study are furnished in this chapter.

3.1 Materials

3.1.1 Plant materials

Brinjal (*Solanum melongena*) wild variety *insanum* I. C. number 421463 reported as resistant to bacterial wilt caused by *Ralstonia solanacearum* and cultivated variety Pusa Purple Long susceptible to aforesaid disease were used in the study. The two varieties selected were observed for important morphological characters. The main characters observed were the plant growth habit, height, plant appearance, spiny nature, leaf, ovary, style and fruit characteristics (Table 1). The varieties were collected from NBPGR, Vellanikkara and IARI, New Dehli. F_1 hybrid progenies of cross *Solanum melongena* var. *insanum* I. C. number 421463 x Pusa Purple Long were raised by controlled crossing. Single F_1 plant was selfed for getting segregating F_2 population for bacterial wilt resistance and for genotyping by RAPD and STS markers.

3.1.2. Laboratory chemicals, glasswares and biological agent

The chemicals used in the present study were of good quality (AR grade) procured from Merck India Ltd., SRL, Sigma, HIMEDIA and SISCO Research Laboratories. The Taq DNA polymerase, RNase, dNTPs, Taq buffer, Agarose, *E. coli* JM-109 strain, Molecular markers (λDNA /*Hind*III +*Eco*RI double digest and 100 bp ladder) were supplied by Bangalore GeNei, pGEM-T Vector System were obtained from Promega Corporation, USA., and DNA Gel Extraction Kit

Table 1. Morphological description of Pusa Purple Long and *Solanum melongena* var. *insanum* I. C. number 421463

Sl. No	Characteristic	Pusa Purple Long	*Solanum melongena* var. *insanum* I.C. number 421463
1	Plant growth	Intermediate	Upright
2	Plant height	80 cm.	1.5 m
3	Plant appearance	spineless, and herbaceous	Spiny, and slightly woody
4	Spiny nature	Posses no spine	Posses spine on stem, leaf and fruit peduncle
5	Width of leaf	3 cm	3.5 cm
6	length of leaf	8 cm	5.5 cm
7	Flower colour	violet	Violet
8	Style	Rarely long	Mostly long
9	Fruit shape	Long	Small rounded
10	Fruit appearance	Glossy, elongated	Non glossy and round
11	Fruit colour	Dark purple	Green with white strips
12	Length of fruit	27.5 cm	5 cm
13	Diameter of fruit	3.5 cm	2.5 cm
14	Quantity seeds	35 nos.	70 nos.
15	Weight of fruit	125g	25g

(Axygen, Biosciences USA) was used for recovery of ready-to-use DNA from gel. The primers were obtained from Operon Technologies, lnc. (Alameda, Calif.). The STS primers were synthesized by Integrated DNA Technologies, USA. The plastic and glass wares used for the study were purchased from Tarsons India Ltd., Axygen, USA and Borosil, India.

3.1.3 Equipment and machineries

The present research work was carried out with the molecular biology facilities and equipments available at CPBMB, College of Horticulture. Centrifugation was done in KUBOTA model No. 65000 centrifuge (Japan), MIKRO 22R (Heittich Zentrifugen) and miniSpin (Eppendorf, Germany). Estimation of quantity and quality of DNA were done by NanoDrop^{R} ND-1000 spectrophotometer (Thermo Scientific, USA.) and horizontal gel electrophoresis systems (Biorad, USA and GeNei India). The PCR was done in the thermal cycler of Mastercycler and Mastercycler gradient (Eppendorf Germany). Gel Documentation System (BIO-RAD Inc. Italy) was used for imaging and documenting the agarose gel. AccuBLOCK ^{TM} Digital Dry Bath model D1100 (Labnet international, Inc USA) was used for making competent cell for transformation. Laminar air flow (THERMODYNE, Faridabad, Haryana; Rotex, B and C Industries, Kerala) was used for maintaining aseptic condition during PCR and transformation. UV transilluminator (WEALTEC, USA) was for viewing and documenting gels with DNA. MILLIPORE (Flix® 3, France) was used for water purification. Shaking Incubator DR-51010 (DAI KI SCINTIFIC, CO. KOREA) was used for multiplication of bacterial cultures. The details are given in Appendix I.

3.2. Methods

3.2.1. Development of Bulk Segregant population

3.2.1.1. Development of F_1 hybrids

The selected varieties *Solanum melongena* var. *insanum* I. C. number 421463 and Pusa Purple Long were grown in pots containing 1:1:1 mixture of sand, soil and FYM. The crop was raised as per package of practices of Kerala Agricultural University (KAU, 2007). The bacterial wilt resistant variety *Solanum melongena* var. *insanum* I. C. number 421463 as female parent was crossed with susceptible variety Pusa Purple Long as male parent for development of F_1 hybrids. Female flower for crossing, was emasculated at 5-6 pm, one day prior to anthesis. Artificial pollination was done early morning between 6 am to 7 am on the day of anthesis. Fruit set was recorded one week after pollination. F_1 plants were produced by sowing seeds from ripe hybrid fruits. Germination percentage of parent seed material and hybrid seeds were recorded.

3.2.1.2. Development of F_2 progenies

The F_2 plants raised from a single F_1 hybrid were used for BSA and STS validation. The selected F_1 plant was selfed to obtain F_2 generation seeds and F_2 generation seeds were sown in pots for development of F_2 progenies.

3.2.2. Phenotyping of genotypes for bacterial wilt resistance

The parental genotypes, F_1 and F_2 population were phenotyped for bacterial wilt resistance. The parental genotypes and F_2 population were phenotyped by artificial inoculation with *Ralstonia solanacearum* inoculum. The F_1 plants were evaluated in sick plot containing rich inoculum.

3.2.2.1. Phenotyping of parents and F_2 population

The seeds of parents and F_2 generation seeds were sown in pots containing sterile potting medium of sand, soil and FYM at the ratio of 1:1:1. Sterilization of

potting medium was carried out with formaldehyde solution prepared by diluting the same with water at ratio of 1:25. Thirty to forty days old seedlings at 3 to 5 leaf stage were transplanted to polythene bag size 32 x 17cm containing sterile potting medium. After 4 days, established seedlings were inoculated with bacterial ooze by stem puncture method. The fresh bacterial ooze from partially wilted brinjal plants was used for inoculation. The bacterial ooze was collected in 100 ml of water and the optical density (OD) of the suspension was adjusted to 0.3 at 600 nm containing 10^8 cfu/ml (James, 2001).

In stem puncture method a drop of bacterial suspension was placed in the axils of second and third expanded leaves below the stem apex and then 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 inoculum. Screening was done during the month of December in open condition. In resistant genotype thirty plants and F_2 forty progenies were evaluated. Plants were observed for 45 days for the incidence of bacterial wilt. The wilted plants were subjected to ooze test to confirm the association of pathogen with the wilt.

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

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

The severity of wilt incidence was scored according to the resistance/susceptibility based on the scoring system by Mew and Ho (1976) and details are given below:-

Disease Rating	**Per cent survival**
Resistant (R)	81 or above
Moderately resistant (MR)	61 – 80
Moderately susceptible (MS)	40 – 60
Susceptible (S)	less than 40

Based on score recorded the genotypes were classified as above.

3.2.2.2. Evaluation of F_1 in Sick plots

Thirty F_1 seedlings were lifted carefully and transplanted in sick plot containing rich population of *Ralstonia solanacearum*. Plants were observed for 65 days for the incidence of bacterial wilt. The wilted plants were subjected to ooze test to confirm the association of pathogen with the wilt. The F_1 population was phenotyped for bacterial wilt resistance as per Mew and Ho (1976).

3.2.3. Molecular characterization of brinjal genotypes by RAPD marker

Molecular characterization of selected resistant parent, susceptible parent, and segregating F_2 progenies were done by RAPD for tagging bacterial wilt resistant gene.

3.2.3.1. Standardization of genomic DNA isolation

Isolation of good quality genomic DNA is one of the most important prerequisite for doing RAPD and STS analyses. The CTAB method reported by Rogers and Bendich (1994) for the extraction of nucleic acids was tested for the isolation of genomic DNA from brinjal with certain modifications. Modifications were made with extraction buffer 1x and washing solution (76% Ethanol/10 mM Ammonium acetate (Grilli *et al.,* 2007). Tender leaves were collected early in the morning and were used for the genomic DNA isolation.

3.2.3.1.2. Procedure CTAB method (Rogers and Bendich, 1994)

Reagents

i. 1x CTAB extraction buffer
ii. 10% CTAB solution
iii. TE Buffer
iv. Isopropanol
v. Chloroform: isopropanol (24:1, v/v)

vi. Ethanol 76%

vii. 10 mM Ammonium acetate

The details of preparation of reagents are provided in Appendix II.

Procedure

I. Leaf sample (1g) was weighed accurately and ground to fine powder in optimum quantity of liquid nitrogen using a pre chilled mortar and pestle in presence of a pinch of poly vinyl pyrrolidine and 50µl of β- mercaptomethanol.

II. Later 3ml extraction buffer was also added. The homogenate was transferred to a 50ml autoclaved oak ridge tube containing 4ml prewarmed lysis buffer. The contents were mixed gently and incubated at 60^0C for 40 minutes in water bath and mixed gently each 5 minutes.

III. Then equal volume of chloroform: isoamyl alcohol mixture was added, mixed gently by inversion and centrifuged at 12,000 rpm for 30 minutes at 4^0C.

IV. The supernatant was transferred to a fresh tube and 1/10 volume of 10% CTAB solution was added. Equal volume of chloroform: isoamyl alcohol was added again, mixed gently and centrifuged at 10,000 rpm for 30 minutes at 4^0C.

V. The aqueous phase was transferred to another fresh tube and added equal volume of chloroform: isoamyl alcohol was added again, mixed gently and centrifuged at 10,000 rpm for 30 minutes at 4^0C.

VI. Then aqueous phase was transferred to another fresh tube. 1/6 volume of chilled isopropanol was added and kept at -20^0C for 1 hour for complete precipitation of DNA.

VII. The DNA was pelleted by centrifuging at 10,000 rpm for 25 minutes at 4^0C.

VIII. The supernatant was discarded and added 0.6 ml washing solution **(**76% Ethanol/10 mM Ammonium acetate Grilli *et al.,* 2007.) into pellet and centrifuge at 10,000 rpm for 3 minutes at 4^0C.

IX. The supernatant was discarded and the pellet was air dried and dissolved in 50-100 µl TE buffer. To facilitate resuspension contents was kept 4^0C for overnight.

X. Then pellet was made to dissolved by tapping and centrifuged at 10,000 rpm for 2 minutes at 4^0C. The contents were then transferred into 1.5 ml autoclaved tube and were stored at -20 ^{0}C.

3.2.3.2. Purification of DNA

The DNA isolated contains RNA as contaminant and was purified by RNase treatment (Nunome *et al.,* 2001.)

Reagents

i. Chilled isopropanol
ii. Ethanol 76%
iii. 10 mM Ammonium acetate
iv. TE buffer
v. RNase

The details of preparation of reagents are provided in Appendix II.

The procedure followed for DNA purification is as follows:

I. RNase solution (2µl) was added to 100µl DNA sample and incubated at 37^0C in water bath for 1 hour.

II. Transferred aqueous phase to autoclaved 1.5ml tube and added 0.6 volume of chilled isopropanol. The mixture was then incubated at -20^{0}C for 1 hour.

III. Then centrifuged at 10,000 rpm for 15 minutes at 4^{0}C. The supernatant was discarded and the added 0.6ml washing solution **(**76 % Ethanol/10 mM Ammonium acetate Grilli *et al.,* 2007) into pellet and centrifuge at 10,000 rpm for 3 minutes at 4^{0}C.

IV. The supernatant was discarded and the pellet was air dried and dissolved in 50-100µl TE buffer.

3.2.3.3. Assessing the quality of DNA by agarose gel electrophoresis

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

Reagents and equipments

i. Agarose
- 0.7 per cent (for genomic DNA)
- 1.4 per cent (for PCR samples)

ii. 50x TAE buffer (pH 8.0)

iii. Tracking dye (6x)

iv. Ethidium bromide

v. Electrophoresis unit, power pack, casting tray,

vi. UV transilluminator

vii. Gel documentation system

(Composition of reagents is provided in Appendix III).

rocedure

The procedure followed for agarose gel electrophoresis is as follows:

1x TAE buffer were prepared from the 50x TAE stock solutions. The open end of gel casting tray was wiped with 100% alcohol and sealed with a cellotape and kept on a horizontal surface. The comb was placed. Agarose (0.7% for DNA) was weighed and dissolved in TAE (1x) buffer by boiling. Then ethidium bromide was added at a concentration of 0.5 μg ml^{-1} and mixed well. Melted 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 well side directed towards the cathode. 1x TAE buffer was added to the tank. Then DNA sample was diluted with water to get approximate terminal concentration of 750ng/ul. Diluted 5ul DNA sample along with the tracking dye 3μl was loaded into the wells using a micropipette carefully. λDNA/ *Eco*RI+*Hind*III double digest was loaded 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 (80V) and current (60 mA) for DNA. The power was turned off when the tracking dye reached more than 2/3rd length for the DNA gel.

Gel documentation

Then the gel was taken from the electrophoresis unit and viewed under UV light in a transilluminator. The DNA fluoresces under UV light due to ethidium bromide dye. The quality of DNA was judged by intactness of DNA band, absence of protein and RNA. The image was documented and saved in gel documentation system.

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

The purity of DNA was further analysed by using NanoDrop® ND-1000 Spectrophotometer. A 2µl sample was used for assessing the purity of DNA. The absorbance of nucleic acid samples were measured at a wavelength of 260 nm and 280nm. The purity of DNA was assessed by the ratio OD_{260}/OD_{280}. A ratio of 1.8 to 2.0 indicated pure DNA. The quantity of DNA in the pure sample was calculated using the formula OD_{260} = 1 is equivalent to 50µg double stranded DNA.

1 OD at 260 nm = 50 µg/ml DNA

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

3.2.3. 5. Isolation DNA for RAPD analysis

DNA for RAPD analysis was isolated as per standardized procedure from resistant parent, susceptible parent and 40 F_2 progenies. DNA isolation of F_2 progenies was done before artificial inoculation for phenotyping them against bacterial wilt incidence. After obtaining the screening data results the DNA of the F_2 progenies were categorized as from resistance and susceptible ones. Isolated DNA was dispersed in TE buffer and stored at -20^0C for further use.

3.2.3.6. RAPD (Random Amplified Polymorphic DNA) analysis

The good quality genomic DNA isolated from brinjal tender leaf by modified CTAB method were subjected to RAPD as per the procedure reported by Williams *et al*. (1990). Random decamer primers supplied by 'Operon Technologies' USA with good resolving power was used for amplification of DNA. So decamer primers for RAPD assay was selected after an initial screening study of primers.

3.2.3.6.1. Screening of random primers for RAPD analysis

Ninety eight decamer primers in the series A, AG, OPA, OPAG, OPAH, OPB, OPC, OPF, OPG, OPH, OPL, OPM, OPP, OPU, PUC, R, RA, RF, RN, RY, S, SC and WG., were screened with genomic DNA of resistant parent *Solanum melongena* var. *insanum* I. C. number 421463 and susceptible parent Pusa Purple Long as the DNA templates and marker. The list of primers with nucleotide sequence is given in Table 2 and 3. The Table 3 represents primers which are reported as wilt specific for solaneacae family members and other crops. Genomic DNA at the concentration of 25-35 ng was subjected to amplification using selected random primers. The amplification reaction was carried out in an Eppendorf Mastercycler and Eppendorf Mastercycler gradient. A master mix without the template DNA was prepared using the reaction mixture for the required number of reactions. From this, master mix, 18µl was pipetted into each PCR tube. Template DNA (2µl) was added. PCR amplification was performed in a 20µl reaction mixture as constituted below:

Composition of the reaction mixture for PCR (20.0 µl)

Genomic DNA (25ng)		2.0µl
10x Taq assay buffer		2.0µl
dNTP mix (10mM each)		1.0µl
$MgCl_2$ (25mM)		1.5µl
Decamer primer (10pM)		1.5µl
Taq DNA polymerase (1U)		0.3µl
Sterile MilliQ water		11.7µl
Total volume	=	20.0µl

Table 2. List of primers used in primer screening

Sl. No.	Name of Primers	Sequence of primers (5'-3')	Sl. No.	Name of Primers	Sequence of primers (5'-3')
1	A-05	AGGGGTCTTG	36	RN-5	ATGCAACGCC
2	A-10	GTGATCGCAG	37	RN-6	ACCAGGGGCA
3	A-12	TCGGCGATAG	38	RN-7	CAGCCCAGAG
4	A-16	AGCCAGCGAA	39	RN-8	ACCTCAGCTC
5	OPA-04	AATCGGGCTG	40	RN-9	TGCCGGCTTG
6	OPA-08	GTGACGTAGG	41	RN-10	ACAACTGGGG
7	OPA-23	AGTCAGCCAC	42	RN-11	TCGCCGCAAA
8	OPA-24	AATCGGGCTG	43	RN-12	CACAGACACC
9	OPA-26	GGTCCCTGAC	44	RN-13	AGCCTCACTC
10	OPA-28	GTGACGTAGG	45	RN-16	AAGCGACTGT
11	OPA-29	GGGTAACGCC	46	RN-18	GGTGAGGTCA
12	OPA-32	TCGGCGATAG	47	RN-19	GTCCGTACTG
13	OPA-34	AATCGCGCAG	48	RN-20	GGTGCTCCGT
14	OPA-38	CAGCGGTGAC	49	RY-01	GTGGCATCTC
15	OPA-40	GTTGCGATCC	50	RY-03	ACAGCCTGCT
16	OPAH-06	GTSSGCCCCT	51	RY-04	GGCTGCAATG
17	OPB-07	GGTGACGCAG	52	RY-06	AAGGCTCACC
18	OPB-15	GGAGGGTGTT	53	RY-07	AGAGCCGTCA
19	OPC-01	TTCGAGCCAG	54	RY-08	AGGCAGAGCA
20	OPC-08	TGGACCGGTG	55	RY-10	CAAACGTGGG
21	OPC-13	GATCCAGCCA	56	RY-11	AGACGATGGG
22	OPC-09	CTCACCGTCC	57	RY-12	AAGCCTGCGA
23	OPC-14	TGCGTGCTTG	58	RY-14	GGTCGATCTG
24	OPF-5	CCGAATTCCC	59	RY-15	AGTCGCCCTT
25	OPF-9	CCAAGCTTCC	60	RY-16	GGGCCAATGT
26	OPP-14	CCAGCCGAAC	61	RY-17	GACGTGGTGA
27	OPU-01	ACGGACGTCA	62	RY-18	GTGGAGTCAG
28	OPU-02	CTGAGGTCTC	63	RY-19	TGAGGGTCCC
29	OPU-03	CTATGCCGAC	64	RY-20	AGCCGTGGAA
30	OPU-09	CCACATCGGT	65	S-02	TGATCCGTGG
31	OPU-13	GGCTGGTTCC	66	S-05	GGACTGGAG
32	OPU-14	TGAAGGGTCA	67	S-15	GGAGGGTGTT
33	RN-1	CTCACGTTGG	68	S-17	AGGGAACGAG
34	RN-3	GGTACTCCCC			
35	RN-4	GACCGACCCA			

Table 3. List of wilt specific primers used in primer screening

Sl No	Primer	Nucleotide sequence (5'-3')	Referances	Crop
1	OPH-02	TCGGACGTGA	Demir *et al.*, 2010	Brinjal
2	OPL-04	GACTGCACAC	Demir *et al.*, 2010	Brinjal
3	OPM-01	GTTGGTGGCT	Bi-hao *et al.*, 2009	Brinjal
4	A-02	TGCCGAGCTG	Nedim *et al.*, 2008	Brinjal
5	PUC-101	AGACCGAACA	Tian *et al.*, 2007	Brinjal
6	GLE-11	CAATCGCCGT	Yang *et al.*, 2006	Brinjal
7	OPAH-09	AGAACCGAGG	Doganler *et al.*, 2002	Brinjal
8	AG-8	AAGAGCCCTC	Osiru *et al.*, 2001	Tomato
9	SC-10:02	GGTCCTCAGG	Mangin *et al.*, 1999	Tomato
10	RA-10:22	GTCGGGTGAA	Yui *et al.*,1999	Tomato
11	RA-12:13	TCATCACACCCG '	Yui *et al.*, 1999	Tomato
12	RA-12:29	AGGTTGGCTGAT	Yui *et al*,1999	Tomato
13	RA-12:38	TGAGAGCGTACG	Yui *et al.*, 1999	Tomato
14	RA-12:41	GACTTGCGCCCA	Yui *et al.*,1999	Tomato
15	RA 12:74	CACTCGATACGG	Yui *et al.*,1999	Tomato
16	RA-12:84	CCCTCCCCAGCT '	Yui *et al.*, 1999	Tomato
17	OPB-17	AGGGAACGAG	Siri *et al.*, 2009	*Solanum commersonii*
18	OPG-03	GAGCCCTCCA	Siri *et al.*, 2009	*Solanum commersonii*
19	OPG-16	AGCGTCCTCC	Siri *et al.*, 2009	*Solanum commersonii*
20	OPH-06	GGAACTCCCC	Siri *et al.*, 2009	*Solanum commersonii*
21	OPH-07	CTGCATCGTG	Siri *et al.*, 2009	*Solanum commersonii*
22	OPH-12	GTGCCTAACC	Siri *et al.*, 2009	*Solanum commersonii*
23	R-6	GGTGGGGACT	Siri *et al.*, 2009	*Solanum commersonii*
24	R-10	CAGCCGCCCC	Siri *et al.*, 2009	*Solanum commersonii*
25	RF	CCCGTCAGCA	Siri *et al.*, 2009	*Solanum commersonii*
26	SC-10:04	CATCGGCGTA	Gousset *et al.*, 2005	*Solanum torvum*
27	SC-10:20	ACTCGTAGCC	Gousset *et al.*, 2005	*Solanum torvum*
28	OPAG-01	CTACGCCTTC	Gangashetti *et al.*, 2004	Rice
29	WG-44	TCGCGCTTTGGA	Onozaki *et al.*, 2004	Carnation
30	WG-54	CCCTCTTGGCTG	Onozaki *et al.*, 2004	Carnation

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

94^0C for 5 minutes	-	Initial denaturation	35 cycles
94^0C for 1 minute	-	Denaturation	
Tm- 3 for 1 minute	-	Primer annealing	
72^0C for 2 minutes	-	Primer extension	
72^0C for 7 minutes	-	Final extension	
4^0C for infinity to hold the sample			

Where-Tm- Melting temperature

The amplified products were run on 1.4 per cent agarose gel using 1x TAE buffer stained with ethidium bromide along with marker (λDNA / *Hind* III + *Eco RI* double digest and 100 bp ladder). The profile was visualized under and documented using gel documentation system. The documented RAPD profiles were carefully examined for polymorphism between template resistant and susceptible parent. Number of bands amplified by each primer were also counted and tabulated. Those primers, which gave polymorphism, were selected for bulked segregant analysis.

3.2.3.6.2. Bulked Segregant analysis of brinjal genotypes

BSA was carried out with resistant parent, susceptible parent, resistant bulk (5 resistant F_2s), susceptible bulk (5 susceptible F_2s), negative control and the marker. Resistant bulk and susceptible bulk consisted of 5 each resistant and susceptible F_2 plants originated from a single F_1 by selfing. Seventeen primers selected after primer screening viz, A-12, OPA-23, OPA-26, OPA-29, OPA-32, OPAH-06, OPC-09, OPP-14, OPU-03, OPU-09, RN-11, RN-12, RN-18, RN-19, RY-01, RY-11, and RY-14 were used for BSA. The sequences of primers used were given in Table 4.

Table 4. List of primers used for bulked segregant analysis

Sl. No.	Name of Primers	Nucleotide sequence (5'-3')
1	A-12	TCGGCGATAG
2	OPA-23	AGTCAGCCAC
3	OPA-26	GGTCCCTGAC
4	OPA-29	GGGTAACGCC
5	OPA-32	TCGGCGATAG
6	OPAH-6	GTSSGCCCCT
7	OPC-09	CTCACCGTCC
8	OPP-14	CCAGCCGAAC
9	OPU-03	CTATGCCGAC
10	OPU-09	CCACATCGGT
11	RN-11	TCGCCGCAAA
12	RN-12	CACAGACACC
13	RN-18	GGTGAGGTCA
14	RN-19	GTCCGTACTG
15	RY-01	GTGGCATCTC
16	RY-11	AGACGATGGG
17	RY-14	GGTCGATCTG

Genomic DNA at the concentration of 35-45ng was amplified using selected random primers. The amplification reaction was carried out in an Eppendorf Mastercycler and Eppendorf Mastercycler gradient. A master mix without the template DNA was prepared using the reaction mixture for the required number of reactions. From this, master mix, 18µl was pipetted into each PCR tube. Template DNA (2µl) was added. PCR amplification was performed in a 25µl reaction mixture as constituted below:

Composition of the reaction mixture for PCR (20.0 µl)

Genomic DNA (42ng)	2.0µl
10x Taq assay buffer	2.5µl
dNTP mix (10mM each)	1.0µl
$MgCl_2$ (25mM)	2.0µl
Decamer primer (10pM)	2.0µl
Taq DNA polymerase (1U)	0.4µl
Sterile MilliQ water	15.1µl
Total volume	= 25.0µl

The PCR tubes were kept in the thermal cycler and were run as per the programme given in section number 3.2.3.6.1.

The amplified products of five groups of genomic DNA for each primer were run on 1.8 per cent agarose gel using 1x TAE buffer stained with ethidium bromide along with λDNA/*Hind*III+*Eco*RI double digest marker and 100 bp ladder. The documented RAPD profile for 17 selected decamer primers were carefully examined for polymorphism among susceptible parent, resistant parent, resistant bulk and susceptible bulk.

The primers which showed polymorphism in resistant and susceptible genotypes were selected. The polymorphic band which was present in resistant

genotypes and absent in susceptible genotypes was eluted for developing STS maker.

3.2.3.6.3. Co-segregation analysis

Individuals of resistant bulk, susceptible bulk, resistant parent and susceptible parent were again screened with RAPD primer OPP-14 which gave phenotype specific bands in resistant parents and resistant bulks using the same PCR ingredients and temperature profile. PCR Products were run in 1.4 per cent agarose gel to see the co-segregation of particular bands with respective phenotypes.

3.2.3.7. Molecular Cloning

3.2.3.7.1. Detection of trait specific markers

The primer OPP-14 gave 470 bp polymorphic band in resistant parent and resistant bulk to bacterial wilt disease. The specific 470 bp amplicon has to be eluted and cloned. For this five RAPD reactions with template DNA from the resistant and susceptible parents were set up as mentioned in section 3.2.3.6.1. One control reaction was also setup with the genomic DNA susceptible parent. After amplification, the products were resolved on 1.4 per cent agarose gel.

3.2.3.7.2. Gel elution of PCR amplicon

The specific DNA band of 470 bp amplified by OPP-14 primer in resistant parent, was cut and lifted from 1.8 per cent agarose gel using sterile scalpel. Gel pieces from three samples were pooled. The DNA of the particular amplicon was eluted using AxyPrep DNA Gel Extraction Kit. Elution was carried out as per the manufacturer's guide lines as shown below:

I. An autoclaved transparent 1.5 ml empty micro centrifuge tube was weighed.

II. DNA fragment of interest was excised from the gel using a sterile, sharp scalpel avoiding much exposure to UV on transilluminator.

III. Gel slice was weighed in the same 1.5 ml micro centrifuge tube.

IV. 3x gel volume of solubilization buffer DE-A (w/v) were added.

V. The gel was resuspended in gel solubilization buffer DE-A by vortexing. It was heated at 75^0C until the gel was completely dissolved. Intermittent vortexing was given every 2-3 minutes to accelerate gel solubilization.

VI. 0.5x gel solubilization buffer volume of binding buffer DE-B was added and mixed properly.

VII. Solubilized gel slice was transferred into the spin column that was assembled in the 2ml collection tube and centrifuged at 12,000 Xg for 1 minute.

VIII. The filtrate was discarded and 500µl of wash buffer (W1) was added to the spin column and centrifuged at 12,000 Xg for 30 seconds.

IX. The filtrate was discarded and 700µl of desalting buffer (W2) was added.

X. Centrifugation was carried out at 12,000 Xg for 30 seconds.

XI. The filtrate was discarded again 700µl of desalting buffer W2 was added.

XII. Centrifugation was carried out at 12,000 Xg for 30 seconds to ensure the complete removal of salt.

XIII. The filtrate was discarded. The spin column was again placed on collection tube.

XIV. Column was again centrifuged for 1 minute at 12,000 Xg to remove any residual buffer.

XV. Spin column was transferred to a fresh autoclaved 1.5ml centrifuge tube. The eluent was prewarmed at 75 ^{0}C to improve the elution efficiency.

XVI. To elute the DNA, 20µl of eluent was added to the centre of the spin column. It was allowed to stand for 1 minute at room temperature. Then it was centrifuged at 12,000 Xg for 1 minute.

XVII. Eluted DNA fragments were checked on 0.7 per cent (w/v) agarose gel, NanoDrop® ND-1000 Spectrophotometer and stored at – 20 ^{0}C.

3.2.3.8. Transformation of *E. coli*

3.2.3.8.1. Preparation of competent cells

Competent cells of *Escherichia coli* JM-109 strain for plasmid transformation were prepared following the protocol of Mandel and Higa (1970).

Medium used were LB medium and LBA medium. (Details of media prepared are given in the Appendix IV).

The steps followed for competent cell preparation were as follows:

Procedure

Day I

The desired *E. coli* strain JM 109 was streaked on LB plate from master plate. The plates were incubated at 37^{o}C for 16 to18 hours.

Day II

I. Inoculated 5 to 6 moderately sized colonies from the LB plate in 25ml LB broth in a 100ml autoclaved test tube.

II. Incubated the broth on a rotary shaker at 37^{o}C; 160 rpm for 3.5 hours. When the OD reached 0.3 at A600, the growth of the bacteria was arrested quickly by chilling for 2 minutes on ice.

III. The entire culture was transferred into a 50ml sterile polypropylene tube and centrifuged at 3,500 rpm for 15 minutes at 4^{o}C.

IV. The supernatant was discarded. Kept the polypropylene tube on ice, the bacterial cell pellet was resuspended very gently in 3.5ml ice cold solution A (0.1 M $CaCl_2$) provided by GeNei.

V. Kept on ice for 20 minutes and then centrifuged at 3,500 rpm for 15 minutes at 4°C.
VI. The supernatant was discarded and the pellet was chilled on ice.
VII. The pellet was resuspended in 600µl of ice cold solution A (0.1 M $CaCl_2$) by blunt ended tip and mixed gently.
VIII. The suspension was left on ice for 10 minutes and aliquots of 100µl were added in chilled 1.5ml microcentrifuge tubes.
IX. The competent cells were covered with aluminium foil and were stored at -70°C.

3.2.3.8.2. Screening of competent cells

Transformation of competent cells with a plasmid pUC18 vector (Fig.1) having ampicillin resistance and *LacZ* gene was carried out to check the competence and purity of competent cells. The procedure followed for screening of plasmid as follows:

I. Prepared 100ml LB solid medium and 50ml LB broth.
II. The cells to be checked for competency stored at -70°C were thawed on ice.
III. Added 1µl of pUC18 from stock to thawed competent cells. Negative control was placed simultaneously without adding plasmid.
IV. The contents were mixed gently and kept on ice for 40 minutes.
V. Meanwhile, a dry bath was set to 42°C.
VI. The tube was rapidly taken from the ice and a heat shock at 42°C was given exactly for 90 seconds. Without shaking, the tube was placed back on ice for 5 minutes.
VII. Added 250µl of LB broth to vial under sterile conditions and was inverted twice to mix the contents. This Mixer was incubated at 37^0C for 1 hour on a shaker set at 150 rpm.

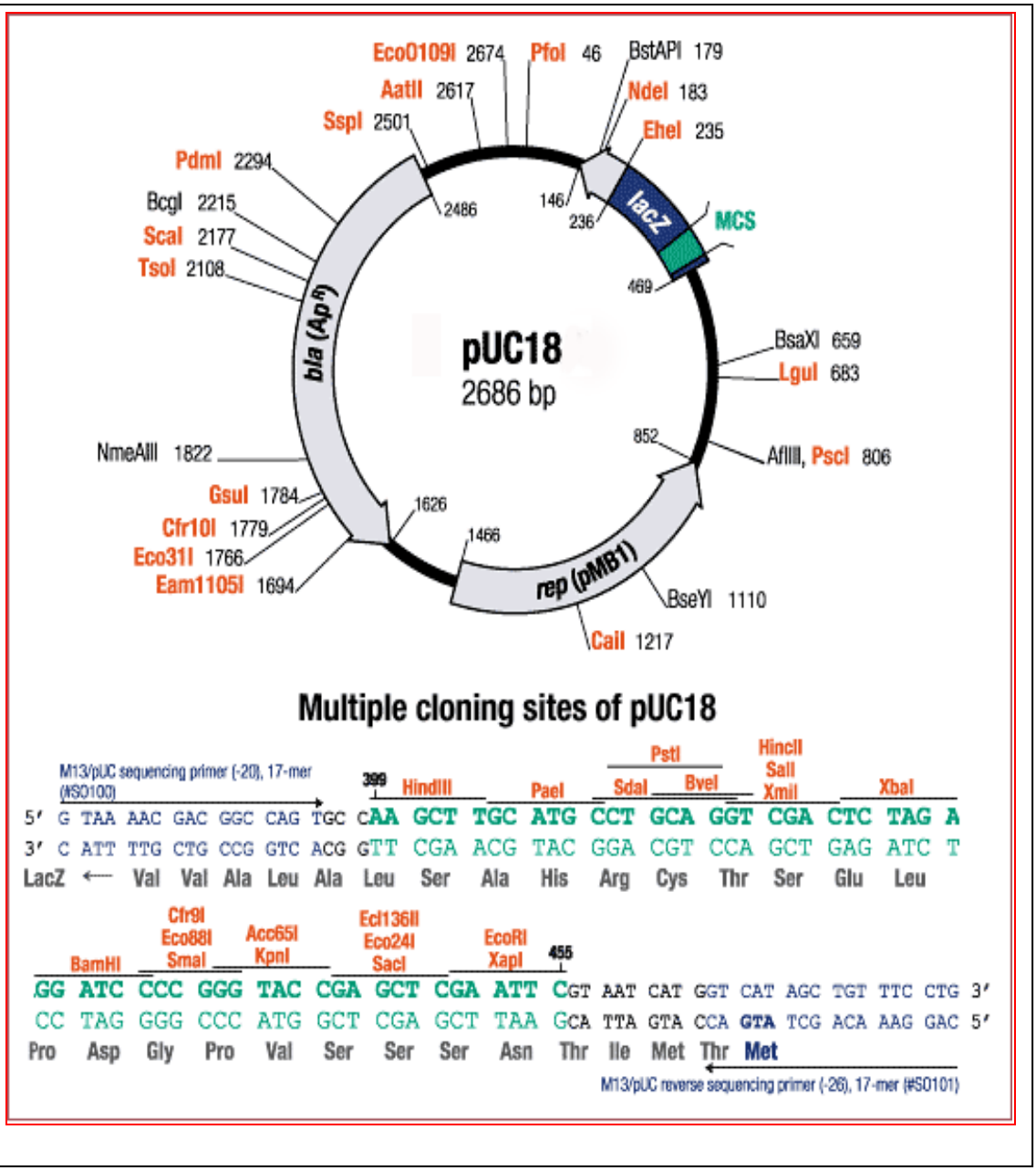


Fig 1. pUC18 Vector System

The *LacZ* region, promoter and multiple cloning sites are shown in the figure.

VIII. 100µl aliquots of the transformed cells were plated on LB ampicillin (100mg/ml) plates layered with IPTG (6µl) and X-gal (12µl) (Detail of master mixer given appendix IV)

IX. Then spread master mix 43µl/ petri plate over solidify LBA ampicillin plate and incubated overnight at 37°C in an incubator.

X. The recombinant clones alone grow on ampicillin plate and colony will be blue coloured.

3.2.3.9. Cloning of eluted DNA

The eluted product was cloned in pGEM-T vector (Fig.2) using pGEM-T Easy Vector System supplied by Promega Corporation, USA.

3.2.3.9.1. Ligation with pGEM-T vector

The Promega's pGEM-T Easy Vector System was supplied after cutting Promega's pGEM-T Vectors with *EcoR V* and adding a 3´ terminal thymidine to both ends. pGEM-T Easy Vector Systems and control insert was centrifuged briefly to collect contents at the bottom of the tubes. The pGEM-T Easy vector contained ampicillin resistance and *LacZ* region as markers. Ligation reaction was set up in 0.5 ml microfuge tubes as follows:

(Procedure followed as per the manufacturer's protocol)

i. Reaction mixture was prepared as described below:

2x rapid ligation buffer	-	5.0µl
pGEM-T Easy Vector (50ng)	-	1.0µl
PCR product (28ng/µl)	-	4.0µl
T4 DNA ligase (3 units/µl)	-	1.0µl
Total volume	=	10µl

ii. The reaction mixture was incubated for one hour at room temperature. Then it was kept at 4^0C overnight. Next day it was taken for transformation in competent cells of *E. coli.*

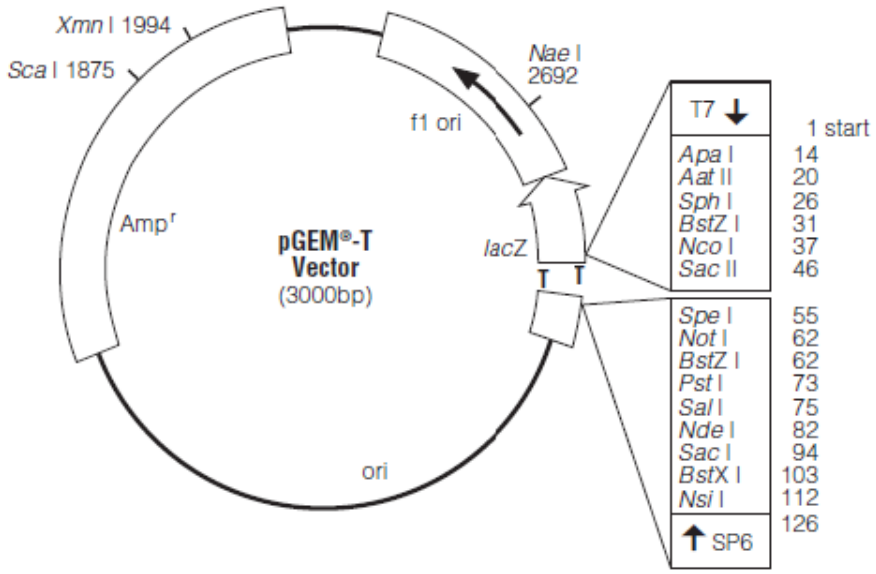


Figure pGEM®-T Vector circle map and sequence reference points.

pGEM®-T Vector Sequence reference points:

T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	126
T7 RNA Polymerase promoter (–17 to +3)	2984–3
SP6 RNA Polymerase promoter (–17 to +3)	124–143
multiple cloning region	10–113
lacZ start codon	165
lac operon sequences	2821–2981, 151–380
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
binding site of pUC/M13 Forward Sequencing Primer	2941–2957
binding site of pUC/M13 Reverse Sequencing Primer	161–177

Fig 2. pGEM-T Vector System

3.2.3.9.2. Cloning of ligated DNA in competent cells

Materials:

I. LB solid medium and LB broth
II. Ligated product
III. Competent cells of *E. coli*
IV. IPTG-0.5 M dissolved in water
V. Ampicillin-10%
VI. X-gal – 10 mg/ml in DMSO

The details of preparation of reagents and medium are provided in Appendix IV.

The procedure followed for transformation of competent cell and blue white screening as follows:

I. The vial containing competent cells was thawed on ice.
II. The ligated product was added to the competent cells, contents mixed gently and kept on ice for 40 minutes.
III. The tube was rapidly taken from ice; heat shock was given at 42^0C exactly for 90 seconds without shaking and placed back on ice for 5 minutes.
IV. Under sterile conditions, 250 µl of LB broth was added and the tube was inverted twice to mix the cells and LB broth.
V. The tube was incubated at 37 ^{0}C for one hour on a shaker setup at 150 rpm.
VI. 100µl aliquots of the transformed cells were plated on LB ampicillin (100 mg/ml) plates layered with IPTG (6µl) and X-gal (12µl) (Detail of master mixer given appendix IV).
VII. Then master mix was spread 43µl/petri plate over solidify LBA ampicillin plate and incubated overnight at 37^{o}C in an incubator.
VIII. The recombinant clones were selected based on blue-white screening.

White colony represent the transformed the cells and blue colony represent the non-transformed ones.

Few blue and white colonies were carefully transferred to the prepared LB/ ampicillin (100mg/ml) plates layered with IPTG (6µl) and X-gal (12µl) grid plates and the colonies were numbered in each plate. The plates were incubated at 37°C overnight and stored at 4°C for further used.

3.2.3.10. Confirmation of the of DNA insert by colony PCR

Colony PCR was carried out with recombinant clones to confirm the presence of inserted DNA by using universal primers T7 forward and SP6 Reverse.

The samples for PCR analysis were the following.

i. DNA of transformed cells from white colony -5 numbers
ii. DNA of non-transformed cells from blue colony- 3 numbers
iii. Positive control for blue colony- 1 number
iv. Positive control for white colony- 2 numbers
v. 100 bp ladder as marker

Conformation of presence of DNA insert was done by comparing molecular weight of different amplicons with marker.

The sample preparation and PCR reaction was setup as follows.

Recombinant bacterial colony was taken by inoculation loop, mixed with 20µl sterile water. Brief centrifugation at 1000 rpm for 30 seconds was given for sedimentation of the bacterial cell constituents. Then DNA was denatured at 94°C for 2 minutes in PCR. 2µl of supernatant was used as a template DNA for amplification of specific DNA insert.

Composition of the reaction mixture for colony PCR

i.	Template DNA	-	2.0µl
ii.	10x Taq assay buffer (A)	-	2.5µl
iii.	dNTP mix (10mM)	-	1.1µl
iv.	Forward primer T7 (1:10)	-	1.0µl
v.	Reverse primer SP6 (1:10)	-	1.0µl
vi.	Taq DNA polymerase (0.3 U)	-	2.0µl
vii.	Sterile MilliQ water	-	15.5µl
	Total volume	=	25.0µl

1. The following PCR programme was run immediately:

94°C for 2 minutes	- Initial denaturation	30 cycles
94° for 45 seconds	- Denaturation	
50°C for 1 minute	- Annealing	
72°C for 1 minute	- Extension	
72°C for 10 minutes	- Final extension	
4°C for 2 minutes	- Cooling of samples	

2. The PCR products from blue white colonies and positive controls for blue, white colonies along with marker were resolved on 2% agarose gel.

3.2.3.11. Preparation of stabs

Pure culture of recombinant *E. coli* was raised in culture tubes. For this the LBA medium containing antibiotic ampicillin (1µl/ml) was melted and poured into storage vial, aseptically and allowed to solidify. Single colony of recombinant bacterial colonies was carefully lifted with a sterile bacterial loop. The loop loaded with bacteria was plunged to the solid medium and incubated the culture tube overnight at 37°C. The stabs showing good growth of bacteria were further stored in refrigerator at 4°C till further use.

3.2.3.12. Sequencing of DNA clones

Stab of recombinant clone named as *W-3* were sent for sequencing to DNA sequencing facility of Bangalore, Chromus Biotech Pvt. Ltd. The primer was T7 and ABI Prism sequence analyzer used for sequencing.

3.2.3.13. *In Silico* analysis of sequences

The sequence information obtained from the Chromus Biotech Pvt. Ltd. Bangalore for *W-3* was further analyzed for its characterization with various online bioinformatics tools.

3.2.3.13.1. Vector screening

The sequences data obtained were subjected to VecScreen tool to identify vector region from sequences of the clones. The VecScreen tool is available in NCBI website (www.ncbi.nlm.nih.gov/VecScreen). The sequences related to vector were removed from the clones using BioEdit Biological sequence alignment editor tool and the remaining sequences were named as "*Sol-3"*.

3.2.3.13.2. Nucleic acid and Protein sequence analysis

The nucleotide sequence of "*Sol-3"* was compared with published sequence in public database using BLAST tool offered by NCBI. Homology search was carried out using Basic Local Alignment Search Tool (BLAST).

The following BLAST programes were used:

1. **Nucleotide- nucleotide BLAST (Blastn)**

The nucleotide sequence of "*Sol-3"* was compared with the sequences available in nucleotide database using Blastn tool (www.ncbi.nlm. nih.gov/blast/; Altschul *et al*., 1997) provided by NCBI.

2. Protein- protein BLAST (Blastp)

The Blastp programme was obtained from (http:// www.ncbi.nm.nih.gov/blast/ and http://www.ebi.ac.uk/Tools/emboss/transeq/ (Altschul et al., 1997). Protein-protein sequence comparison was done using Blastp. The best sequence alignment results were noted and saved.

3. Detection of Open Reading Frame (ORF)

To find the open reading frame of the "*Sol-3"* nucleotide sequence, the programme 'ORF finder' (www.ncbi.nlm.nih.gov/gorf/gorf.html) of NCBI was used. The best ORF results were noted and saved.

3.2.4. Development of STS marker

3.2.4.1. STS primer designing:

For designing longer primers of 22 bp length the sequence of cloned RAPD fragment 0PP-14 "*Sol-3"* was used.

The following criteria were considered during STS primer designing:

I. The end sequences selected should have GC content 40-50 per cent.
II. Melting temperature (Tm = 4 GC + 2 AT) ranged between 52^0C to 58^0C.
III. The distance between the primers ranged from 200 to 400 base pairs.
IV. It is preferable to have GC content at 3' end.
V. There should not be any complementarity between forward and reverse primers.
VI. Repeats of single base should not appear within the primer sequence.
VII. The distance between forward and reverse primer should be greater than 200 bp.
VIII. Each primer should be 22 to 27 bp long.

IX. Primer sequences should not have palindromic sequences or repetitive sequences.

X. For designing primers, the sequence of the forward primer was taken as such and for the reverse primer, the reverse complementary sequence was taken.

Based on above information, nucleotide sequence of forward and reverse STS primers was designed by using online Primer3Plus tool. The forward and reverse primers were selected from the RAPD fragment regions and they were named as BWRGB-1 and BWRGB-2 sets.

3.2.4.3. PCR analysis with STS primer

The DNA of resistant parent, susceptible parent, resistant bulk and susceptible bulk were amplified using STS primer BWRGB-2. A negative control was also kept without template DNA. PCR was carried out in Mastercycler as per the reaction mixture and programme given below. The annealing temperature was standardized in gradient PCR.

Composition of the reaction mixture for PCR analysis was the following:-

Genomic DNA (45ng)	-	2.5µl
10x Taq assay buffer	-	2.5µl
$MgCl_2$ (25mM)	-	2.0µl
dNTP mix (10mM)	-	1.2µl
Forward primer (2:10)	-	2.5µl
Reverse primer (2:10)	-	2.5µl
Taq DNA polymerase (0.3 U)	-	0.4µl
Sterile MilliQ water	-	11.4µl
Total volume	=	25.0 µl

PCR programme

94 ^{0}C	for 5 minutes - Initial denaturation	
94 ^{0}C	for 1 minute - Denaturation	
58 ^{0}C	for 1 minute – Annealing	35 cycles
72 ^{0}C	for 2 minutes - Extension	
72 ^{0}C	for 7 minutes - Final extension	
4 ^{0}C	for 2 minutes to hold the sample	

The PCR product was resolved on 2% agarose, documented and interpreted.

Results

4. RESULTS

The results of the investigations conducted on the "Tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* by molecular markers" undertaken during the period from 2009 to 2011 at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara, are described in this chapter.

4.1. Plant materials

The two selected varieties Pusa Purple Long and *Solanum melongena* var. *insanum* I. C. number 421463 were observed for important morphological characters after flowering and fruits setting and observations are presented in Table 1. In plant growth habit Pusa Purple Long is intermediate with mean height of 80 cm. and *insanum* is upright with mean height of 1.5 m. In appearance Pusa Purple Long was herbaceous and spineless. *Insanum* was woody with spine all over stem, leaf and fruit peduncle. Leaf width was 3 cm in Pusa Purple Long while it was 3.5 cm in *insanum*. Leaf length was more in Pusa Purple Long, 8 cm while it was less in *insanum*, 5.5 cm. Flower colour was violet in both the genotypes. Style rarely long in Pusa Purple Long while mostly long in *insanum*. Length of fruit 27.5 cm in Pusa Purple Long while 5 cm *insanum*.

4.2. Development of Bulked segregating generation

Crosses were made using the resistant variety *Solanum melongena* var. *insanum* I. C. number 421463 as the female parent (Plate 1a) and susceptible variety Pusa Purple Long as the male parent (Plate 1b). The fruit setting per cent in this cross was 4 per cent. F_1 plants were generated (Plate 1c) from F_0 seeds. Forty F_2 plants (Plate 1d) were raised from a single F_1 plants by selfing and they were used as segregating population for tagging the bacterial wilt resistant gene. The fruit setting in F_1 plants was 11 per cent when selfed.

X

a) Resistant parent: *Solanum melongena* var. *insanum* I. C. No. 421463

b) Susceptible Parent: Pusa Purple Long

c) F_1 Hybrid: *Solanum melongena* var. *insanum* I. C. No. 421463 X Pusa Purple Long

d) F_2 Progeny: *Solanum melongena* var. *insanum* I. C. No. 421463 X Pusa Purple Long

Plate 1. Development of F_1 and F_2 progenies

Germination percentage in *Solanum melongena* var. *insanum* I. C. number 421463 was 2 per cent, F_0 hybrids seeds was 80 per cent and F_1 generation seeds was 80 per cent.

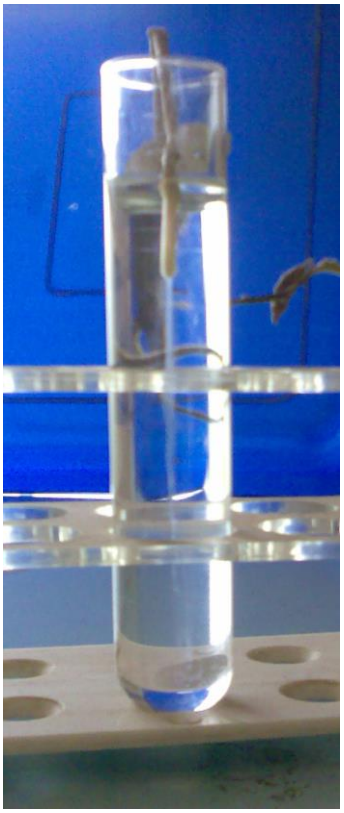
4.3. Phenotyping of genotypes for bacterial wilt resistance

4.3.1. Phenotyping of parents and F_2 population

Forty F_2 progenies along with the resistant parent and susceptible parent were screened by artificial inoculation technique. Artificial inoculation was given by stem-puncture method. The wilt symptoms were observed 7 days after inoculation in susceptible parent under open condition. In resistant parent symptoms appeared after 15 days. The symptom started as leaf drooping followed by complete wilting of the plant (Plate 2a). The wilted plants showed positive response to bacterial ooze test (Plate 2b). The plants were observed for 45 days and were scored as resistant or susceptible based on survival. In each genotype percentage of plants survived were found out and were categorized as moderately resistant (MR) and susceptible (S) as per the scoring system of Mew and Ho (1976).

The survival percentage *Solanum melongena* var. *insanum* I. C. number 421463 was 85 per cent and was scored as resistant. The Pusa Purple Long was scored as susceptible (S) as survival percentage was zero. The F_2 population was categorized as susceptible (S) with 27 per cent survival. Based on screening data of F_2 population five each susceptible and resistant progenies were selected for bulked segregant analysis.

a) Complete wilting of brinjal plants



b) Bacterial Ooze test

Plate 2. Symptom of bacterial wilt and its confirmation

4.3.2. Evaluation of F_1 in Sick plots

Thirty F_1 progenies were screened in sick plots for bacterial wilt incidence. The wilt symptoms were observed 30 days after transplanting. The wilted plants showed positive response to bacterial ooze test (Plate 2b). They were observed for 65 days and were scored as resistant or susceptible based on survival. F_1 progenies recorded survival percentage of 80 and were categorized as moderately resistant (MR) as per the scoring system of Mew and Ho (1976).

4.4. Molecular characterization of brinjal genotypes with RAPD marker

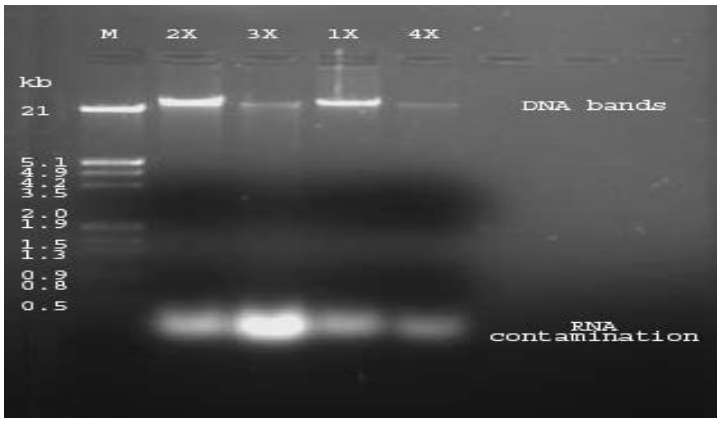
Molecular characterization of selected brinjal genotypes and F_2 population for tagging bacterial wilt resistant gene was done with RAPD marker (Plate 1).

4.4.1. Standardization of genomic DNA isolation

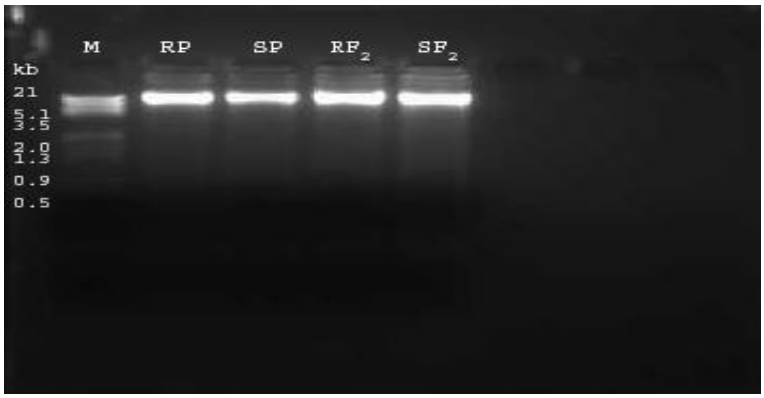
Tender, immature pale green leaves collected early in the morning were used for the genomic DNA isolation. The CTAB method reported by Rogers and Bendich, 1994 with some modifications yielded DNA from tender leaves. The extracted DNA was electrophoresed on agarose gel. Use of 1x extraction buffer and washing solution of 76 per cent ethanol/containing 10mM Ammonium acetate (Grilli *et al.*, 2007) yielded good quantity DNA with little RNA contamination (Plate 3a). Use of extraction buffers 2x, 3x and 4x yielded poor quality DNA with high RNA contamination. They were free of protein contamination.

4.4.2. Purification of DNA

The genomic DNA isolated by modified Roger and Bendich (1994) method showed RNA contamination in few DNA samples. So the DNA isolated by this method was purified by RNase treatment (Nunome *et al.*, 2001.). RNase removed RNA contamination in all DNA samples (Plate 3b). The DNA isolated by this method appeared as clear and single intact band of 15kb with high intensity.



a) DNA isolated by CTAB method by 1x, 2x, 3x and 4x extraction buffer



b) DNA isolated by CTAB method 1x after RNase treatment

Plate 3. Standardization of genomic DNA isolation and purification

4.4.3. Assessing the quality of DNA by agarose gel electrophoresis

The quality and quantity of DNA isolated using the aforesaid protocols were assessed using agarose gel electrophoresis. The DNA isolated by this method appeared as clear and single intact band of high intensity with molecular weight 15 kb (Plate 3b). This indicated non-degraded good quality DNA free from RNA contamination.

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

The quality and quantity of DNA thus isolated was analyzed using NanoDrop® ND-1000 spectrophotometer and the results are presented in Table 5. The ratio of UV absorbance ($A_{260}/_{280}$) ranged between1.85- 1.95. Since ratio of UV absorbance ($A_{260}/_{280}$) comes between 1.85- 1.95 quality of DNA was rated as good (Table 5). The quantity of DNA in the isolated samples ranged from 3163.93 to 4668.11 ng/µl.

4.4.5. Isolation DNA for RAPD analysis

DNA for RAPD analysis was isolated as per standardized procedure from resistance parent, susceptible parent and 40 F_2 progenies. DNA isolation of F_2 progenies was done before artificial inoculation for phenotyping them against bacterial wilt incidence. After obtaining the screening data results, the DNA of the F_2 progenies were categorized as resistant and susceptible ones. The quality and quantity of DNA samples *viz*, resistant parent (RP), susceptible parent (SP), five resistant F_2 progenies (RF_2 1-5) and five susceptible F_2 progenies (SF_2 1-5) are presented in Table 6. The ratio of UV absorbance ($A_{260}/_{280}$) ranged between 1.80-1.99 in resistant parent, susceptible parent and F_2 progenies. The quantity of DNA in the isolated samples ranged from 3794.49 to 4702.84ng/µl. The DNA, thus isolated, after 25-45ng/ul dilutions were used as templates for RAPD and STS analysis. Isolated DNA was dispersed in TE buffer and stored at -20^0C for further use.

Table 5. Quality and quantity of DNA isolated from brinjal genotypes assesed by NanoDrop method

Genotype	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260/280}$	Quantity (ng/µl)	Quality
RP	93.362	50.406	1.85	4668.11	Good
SP	63.279	32.413	1.95	3163.93	Good

Table 6. Quality and quantity of DNA isolated from parents and F_2 progenies assesed by NanoDrop method

Genotype	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260/280}$	Quantity (ng/µl)	Quality
RP	93.715	50.416	1.86	4685.73	Good
SP	84.982	43.904	1.95	4249.11	Good
RF_2 1	83.409	42.253	1.97	4170.43	Good
RF_2 2	94.057	51.769	1.82	4702.84	Good
RF_2 3	75.890	37.881	2.00	3794.49	Good
RF_2 4	81.725	41.412	1.97	4086.24	Good
RF_2 5	93.076	49.915	1.86	4653.81	Good
SF_2 1	78.756	39.570	1.99	3937.78	Good
SF_2 2	91.370	49.348	1.85	4568.49	Good
SF_2 3	83.409	42.253	1.97	4170.43	Good
SF_2 4	84.394	43.584	1.94	4219.70	Good
SF_2 5	75.890	37.881	2.00	3794.49	Good

RP: Resistant parent

SP: Susceptible parent

RF_2- resistant F_2 s

SF_2-susceptible F_2 s

4.5. 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, bulked segregant analysis and co-segregation of brinjal genotypes using selected primers.

4.5.1. Screening of primers for RAPD analysis

Ninety eight primers belonging to 23 Operon series viz., A, AG, OPA, OPAG, OPAH, OPB, OPC, OPF, OPG, OPH, OPL, OPM, OPP, OPU, PUC, R, RA, RF, RN, RY, S, SC and WG were screened to test their ability to produce polymorphism between resistant and susceptible genotypes. The number of amplicons produced by each primer was also examined. The list of primers with nucleotide sequence is given in Table 2 and 3. The Table 3 represents primers which are reported as wilt specific for solanaceae family members and other crops.

4.5.1.1. Screening with A series

Four primers A-05, A-10, A-12 and A-16 were screened in this series. The amplification pattern observed for different primers in A series is given in Table 7 and Plate 4a and 4b, respectively. Mean number of amplicons ranged between 1 to 11 in A-16 and A-10. In this series primer A-12 has given polymorphism and polymorphic bands were of size 0.8 and 1.9 kb. This primer has amplified 11 each amplicons in resistant and susceptible parent. The primer A-12 was selected for BSA based on polymorphism and good amplification.

4.5.1.2. Screening with OPA series

Eleven primers in this series viz., OPA-04, OPA-08, OPA-23, OPA-24, OPA-26, OPA-28, OPA-29, OPA-32, OPA-34, OPA-38 and OPA-40 were screened. The amplification pattern observed for different primers in OPA series

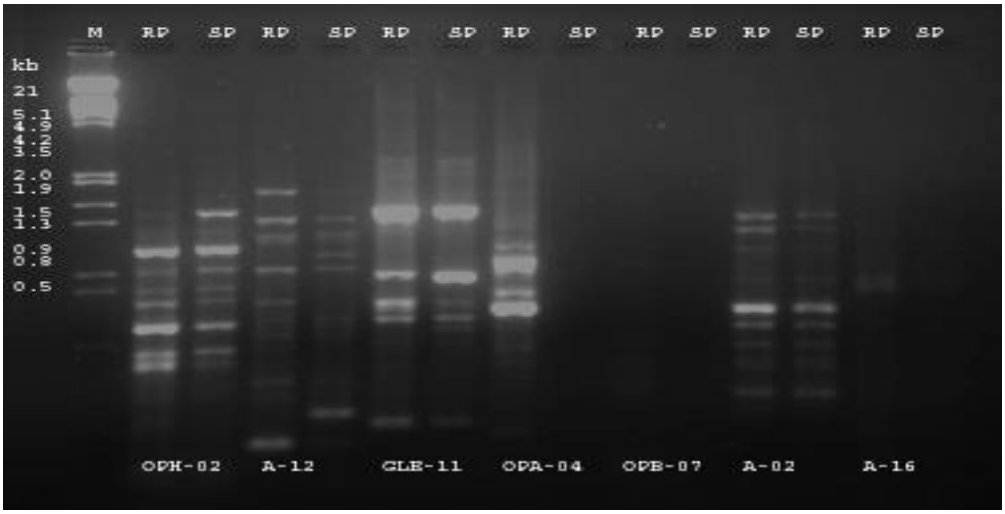
Table 7. Amplification patterns of brinjal genotypes with A series primers

Sl. No.	Name of Primers	No. of bands		Mean	No. and molecular weight of polymorphic bands		Amplification pattern
		RP	SP			Kb.	
1	A-05	6	6	6	0		Good
2	A-10	9	11	10.5	0		Good
3	A-12	11	11	11	1	0.8	Good
4	A-16	1	1	1	0		Poor

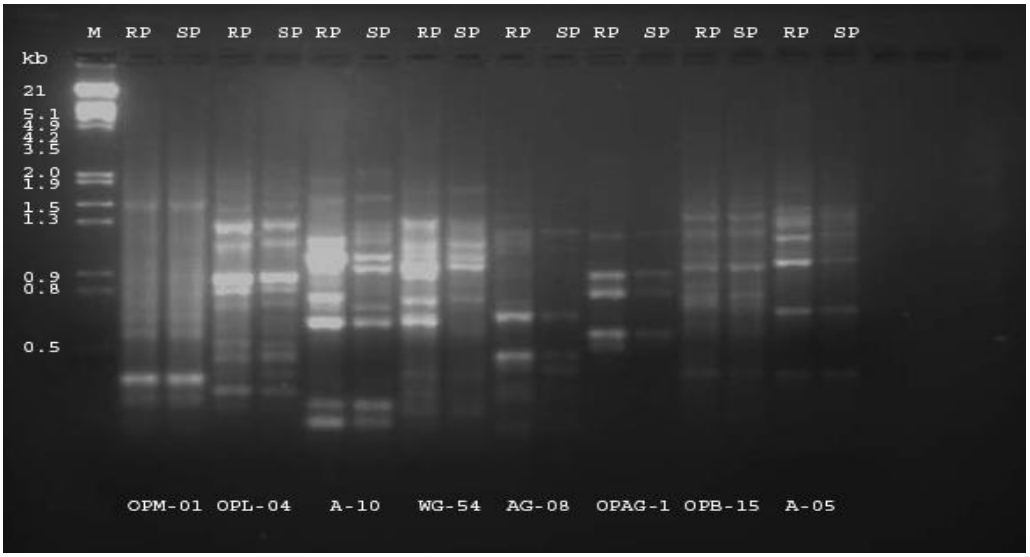
Table 8. Amplification patterns of brinjal genotypes with OPA series primers

Sl. No.	Name of Primers	No. of bands		Mean	No. and molecular weight of polymorphic bands		Amplification pattern
		RP	SP			Kb.	
1	OPA-04	7	8	7.5	0		Good
2	OPA-08	0	0	0	0		No amplification
3	OPA-23	8	6	7	2	0.8,0.6	Good
4	OPA-24	6	7	6.5			Good
5	OPA-26	8	5	6.5	4	0.8,0.7, 0.4, 0.3	Good
6	OPA-28	9	9	9	0		Good
7	OPA-29	10	7	8.5	1	0.5	Good
8	OPA-32	9	5	7	2	0.5, 0.2	Good
9	OPA-34	0	0	0	0		No amplification
10	OPA-38	1	1	1	0		Poor
11	OPA-40	7	7	7	0		Good

RP: Resistant parent SP: Susceptible parent



a)



b)

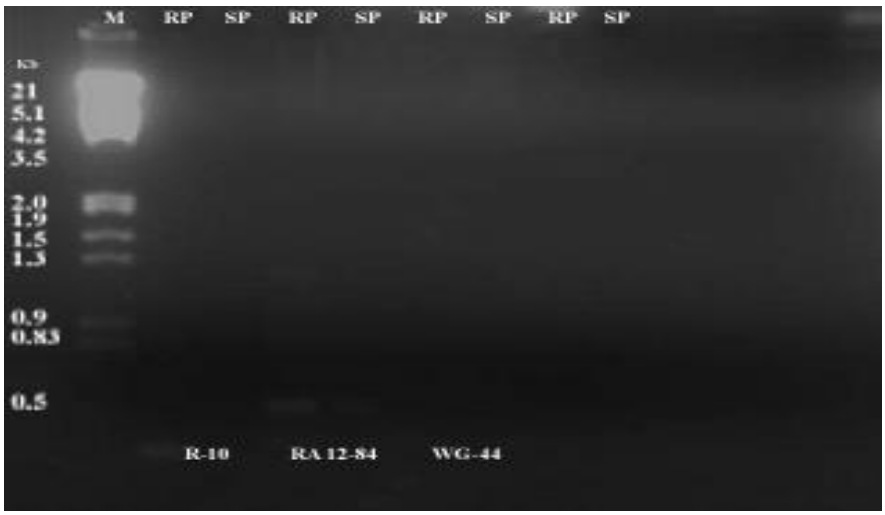
M: Marker λ DNA / *EcoRI/ HindII*

RP: Resistant parent, SP: Susceptible parent

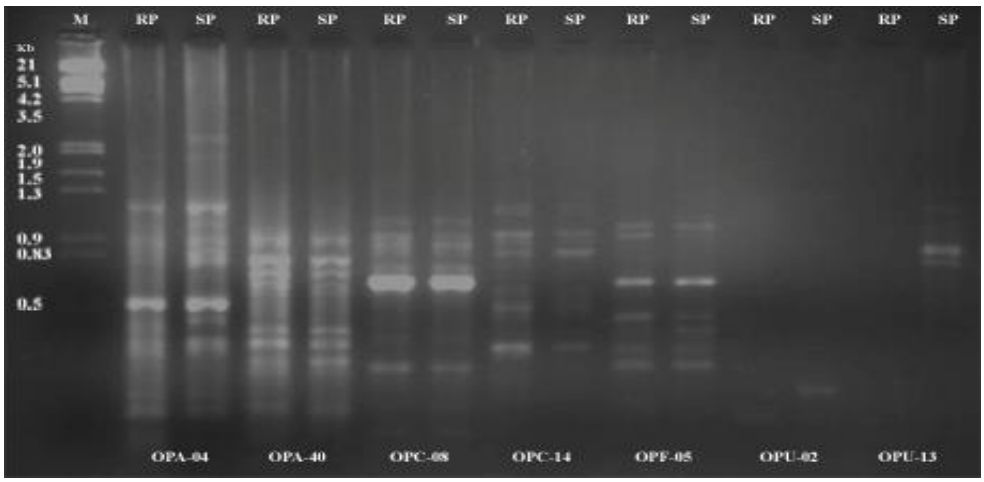
a) OPH-02, A-12, GLE-11, OPA-04, OPB-07, A-02 and A-16

b) OPM-01, OPL-04, A-10, WG-54, AG-08, OPAG-1, OPB-15 and A-05

Plate 4. Screening of primers of OPH, A, GLE, OPA, OPB, OPM, OPL, WG, AG and OPAG series with resistant and susceptible parents.



a)



b)

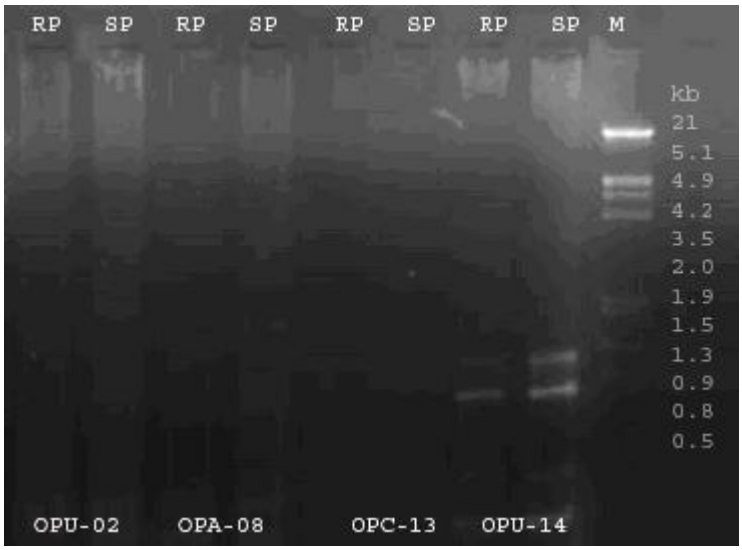
M: Marker λ DNA / *EcoRI/ HindII*

RP: Resistant parent, SP: Susceptible parent

a) R-10, RA-12:84 and WG-44

b) OPA-04, OPA-40, OPC-08, OPC-14, OPF-05, OPU-02 and OPU-13

Plate 5. Screening of primers of R, RA, WG, OPA, OPC, OPF and OPU series with resistant and susceptible parents.



M: Marker λ DNA / *EcoRI/ HindII*

RP: Resistant parent, SP: Susceptible parents

OPU-02, OPA-08, OPC-13 and OPU-14

Plate 6. Screening of primers of OPU-02, OPA-08, OPC-13 and OPU-14 primers with resistant and susceptible parents.

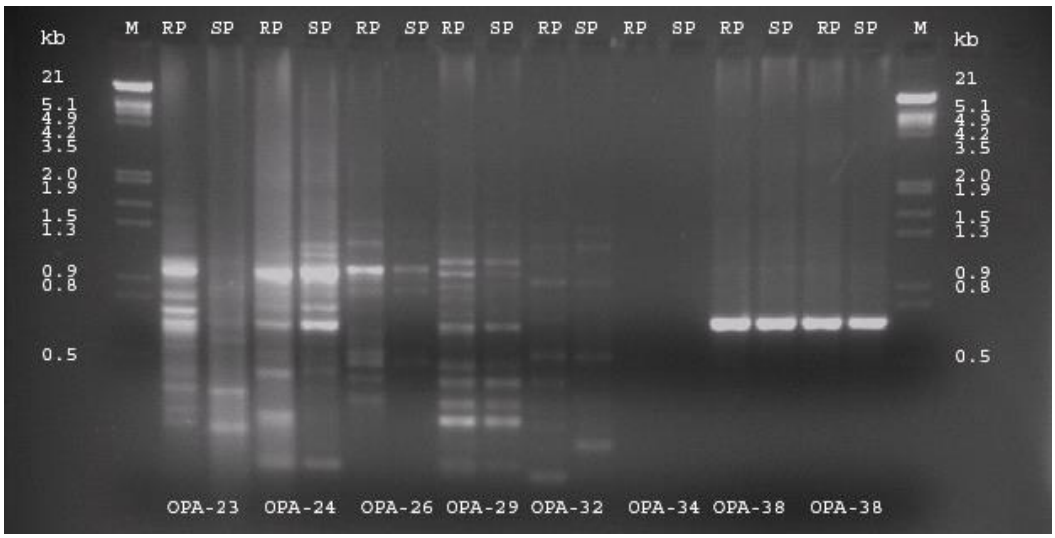
is given in Table 8 and Plate 5b, 6, 7a, 7a, 7a, 7b, 7a, 7a, 7a, 7a and 7b, respectively. The mean number of bands in this series varied from 0 to 9. The primers which had given polymorphism are OPA-23, OPA-26, OPA-29 and OPA-32. The primer OPA-23 has given nine each amplicons in resistant and susceptible parent. Two polymorphic bands of 0.8 and 0.6 kb were present resistant parent. The primer OPA-26 has given 9 and 5 bands in resistant and susceptible parent respectively. It has produced two polymorphic bands of 1.3 and 0.5 kb in resistant parent. The primer OPA-29 has given 10 and 8 bands in resistant and susceptible parent respectively. It has produced one polymorphic band of 0.5 kb in resistant parent. The primer OPA-32 has given 9 and 5 bands in resistant and susceptible parent respectively. It has produced two polymorphic bands of size 0.7 and 0.4 kb in resistant parent. The primers OPA-23, OPA-26, OPA-29, and OPA-32 were selected for BSA based on polymorphism and good amplification.

4.5.1.3. Screening with OPB series

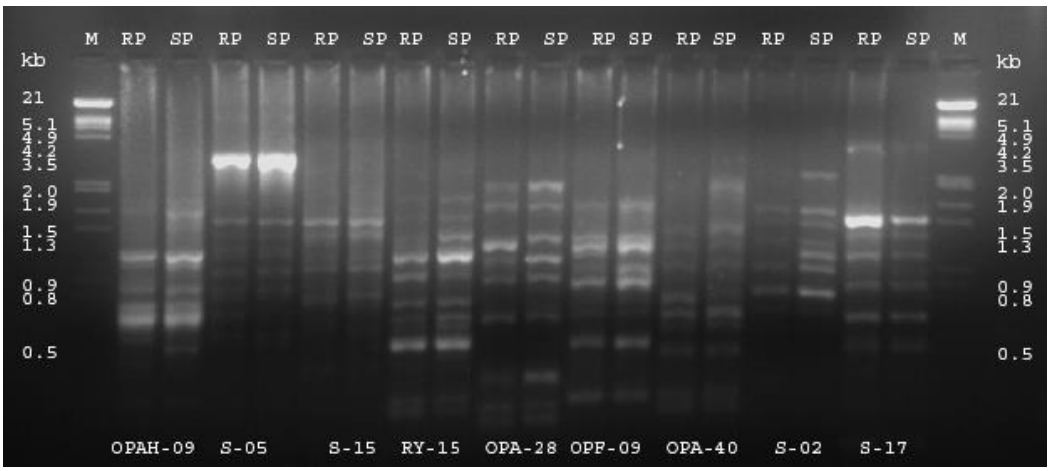
Two primers OPB-07 and OPB-15 in this series were screened. The amplification pattern observed for OPB series is given in Table 9 and Plate 4a and 4b, respectively. OPB-07 did not amplify any DNA. The primer OPB-15 produced a mean 5.5 numbers of bands in resistant and susceptible parent. These primers were not selected for BSA.

4.5.1.4. Screening with OPC series

Five primers in this series viz., OPC-01, OPC-08, OPC-09, OPC-13 and OPC-14 were screened. The amplification pattern observed for OPC series is given in Table 9 and Plate 8a, 5b, 8a, 6 and 5b, respectively. The primer has given 4 bands in resistant parent and 6 bands in susceptible parent. It has given polymorphic band of 0.5 kb in resistant parent. The primer OPC-09 was selected for BSA based on polymorphism and good amplification.



a)



b)

M: Marker λ DNA / *EcoRI/ HindII*

RP: Resistant parent, SP: Susceptible parent

a) OPA-23, OPA-24, OPA-26, OPA-29, OPA-32 and OPA-38

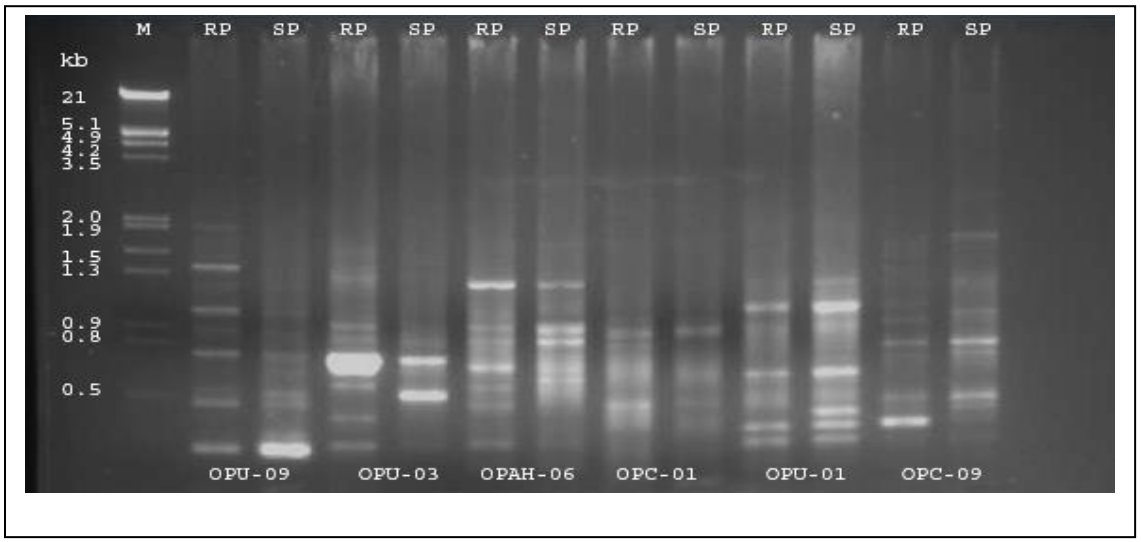
b) OPAH-09, S-05, S-15, RY-15, OPA-28, OPF-09, OPA-40, S-02 and S-17

Plate 7. Screening of primers of OPA, OPAH, S, RY and OPF series with resistant and susceptible parents.

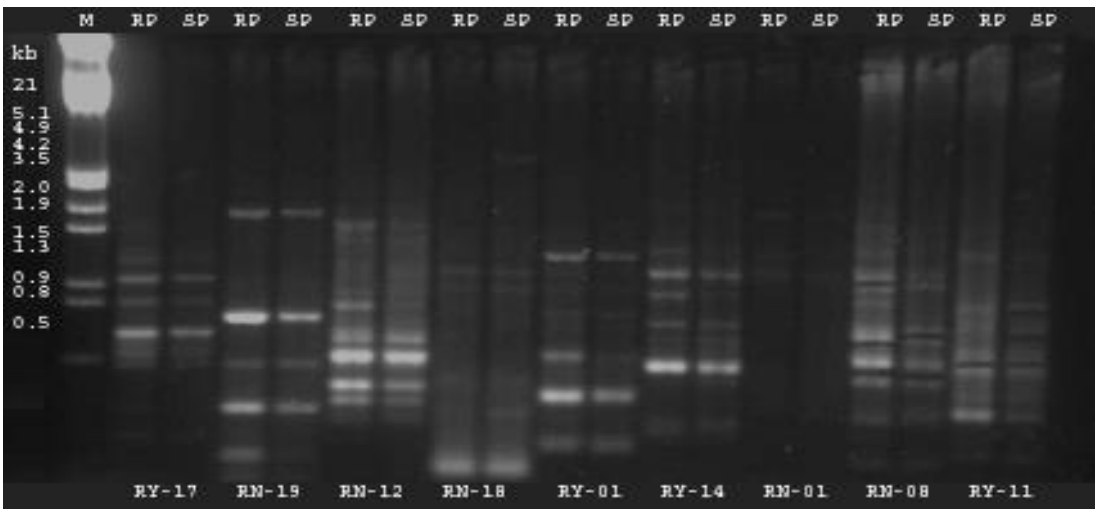
Table 9. Amplification patterns of brinjal genotypes with OPAH, OPB, OPC, OPF, OPP, and OPU series primers

Sl. No.	Name of Primers	No. of bands		Mean	No. and molecular weight of polymorphic bands		Amplification pattern
		RP	SP			Kb.	
1	OPAH-06	7	5	6	2	0.6, 0.4	Good
2	OPB-07	0	0	0	0		No amplification
3	OPB-15	6	6	6	0		Good
4	OPC-01	5	5	5	0		Medium
5	OPC-08	5	5	5	0		Medium
6	OPC-09	5	7	6	1	0.5	Good
7	OPC-13	0	0	0	0		Poor
8	OPC-14	7	7	7	0		Good
9	OPF-05	6	6	6			Good
10	OPF-09	7	7	7	0		Good
11	OPP-14	5	4	4.5	1	0.45	Medium
12	OPU-01	4	5	4.5	0		Medium
13	OPU-02	0	0	0	0		No amplification
14	OPU-03	7	3	5	4	0.9,0.7, 0.5, 0.3	Medium
15	OPU-09	7	5	6	2	1.3, 0.9	Good
16	OPU-13	0	0	0	0		No amplification
17	OPU-14	3	4	3.5	0		Poor

RP: Resistant parent SP: Susceptible parent



a)



b)

M: Marker λ DNA / *EcoRI/ HindII*

RP: Resistant parent, SP: Susceptible parent

a) OPU-09, OPU-03, OPAH-06, OPC-01, OPU-01 and OPC-09

b) RY-17, RN-19, RN-12, RN-18, RY-01, RY-14, RN-01, RN-08 and RY-11

Plate 8. Screening of primers of OPU, OPAH, OPC, OPU, RY and RN series with resistant and susceptible parents.

4.5.1.5. Screening with OPF series

Two primers OPF-05 and OPF-09 in this series were screened. The amplification pattern observed for OPF series is given in Table 9 and Plate 5b and 7b, respectively. They have obtained an average 5 and 7 amplicons in resistant and susceptible parent. The OPF series primers were not selected for BSA as there was no polymorphism.

4.5.1.6. Screening with OPU series

Six primers OPU-01, OPU-02, OPU-03, OPU-09, OPU-13 and OPU-14 were screened. The amplification pattern observed for these primers is given in Table 9 and Plate 8a, 6, 8a, 8a, 5b and 6, respectively. The primer OPU-03 has given on an average 4.5 bands in resistant and susceptible parent. It has also generated two polymorphic bands of 1 and 0.4 kb in resistant parent. The primer OPU-09 has given an average 6 bands in resistant and susceptible parent. It has produced one polymorphic band of 1 kb in resistant parent. The primers OPU-03 and OPU-09 were selected for BSA based on polymorphism and good amplification.

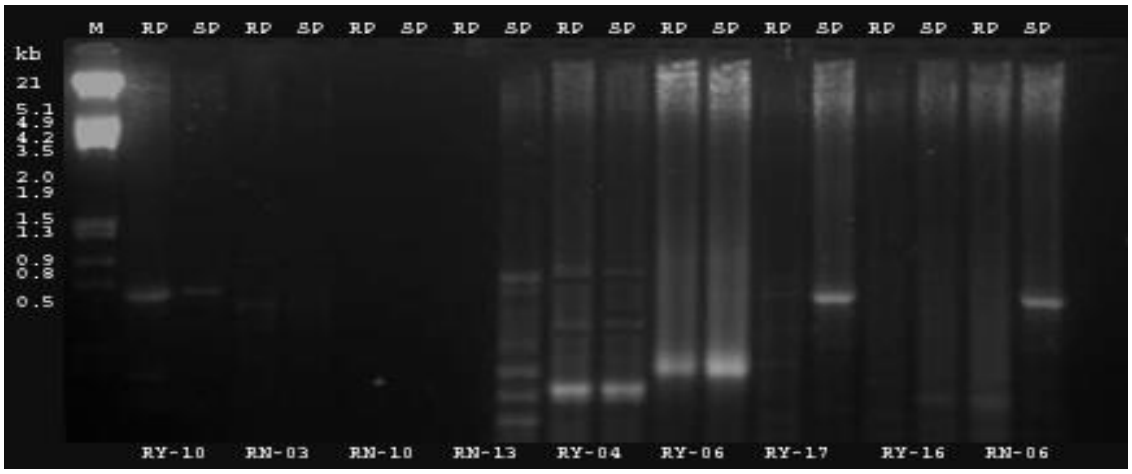
4.5.1.7. Screening with RN series

Sixteen primers, RN-01, RN-03, RN-04, RN-05, RN-06, RN-07, RN-08, RN-09, RN-10, RN-11, RN-12, RN-13, RN-16, RN-18, RN-19 and RN-20 in this series were screened. The amplification pattern observed for these primers is given in Table 10 and Plate 8b, 9a, 10b, 10a, 10a, 10a, 8b, 10b, 9a, 10b, 8b, 9a, 10a, 8b, 8b, and 10b, respectively. The mean number of bands in this series varied from 0 to 8.5. The primers which had given polymorphism are RN-11, RN-12, RN-18, and RN-19. The primer RN-11 has given six each amplicons in resistant and susceptible parent and one polymorphic band of 1.3 kb in resistant parent. The primer RN-12 has given 7 and 6 bands in resistant and susceptible parent respectively. It has produced one polymorphic band of 0.8 kb in resistant parent. The primer RN-18 has given five each bands in resistant and susceptible parent.

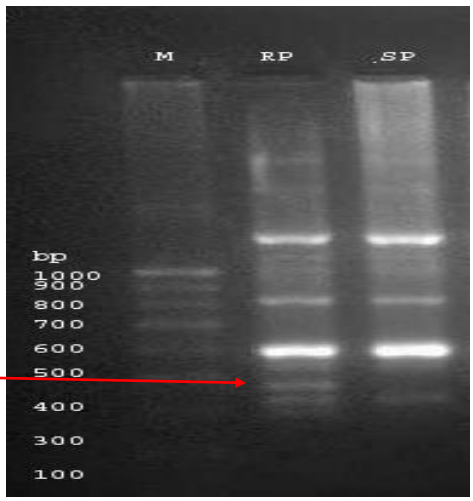
Table 10. Amplification patterns of brinjal genotypes with RN series primers

Sl. No.	Name of Primers	No. of bands		Mean	No. and molecular weight of polymorphic bands		Amplification pattern
		RP	SP			Kb.	
1	RN-01	0	0	0	0		No amplification
2	RN-03	0	0	0	0		No amplification
3	RN-04	4	2	3	0		Poor
4	RN-05	2	9	5.5	0		Medium
5	RN-06	6	6	6	0		Good
6	RN-07	5	9	7	0		Good
7	RN-08	6	6	6	0		Good
8	RN-09	4	2	3			Poor
9	RN-10	0	0	0	0		No amplification
10	RN-11	5	2	3.5	1	1.3	Medium
11	RN-12	6	4	5	1	0.8	Medium
12	RN-13	5	5	5	0		Medium
13	RN-16	9	9	9	0		Good
14	RN-18	5	4	4.5	1	0.4	Medium
15	RN-19	6	5	5.5	1	0.3	Good
16	RN-20	5	3	4			Medium

RP: Resistant parent SP: Susceptible parent



a)



470 bp Polymorphic band

b)

M: Marker λ DNA / *EcoRI/ HindII* and 100 bp ladder

RP: Resistant parent, SP: Susceptible parent

a) RY-10, RN-03, RN-10, RN-13, RY-04, RY-06, RY-17, RY-16 and RN-06

b) OPP-14

Plate 9. Screening of primers of RY, RN series and OPPP-14 with resistant and susceptible parents.

The primer RN-18has given five each bands in resistant and susceptible parent.It has produced one polymorphic band of 0.5 kb in resistant parent. The primer RN-19 has given 6 and 5 bands in resistant and susceptible parent respectively. It has produced one polymorphic band of size 0.3kb in resistant parent. The primers RN-11, RN-12, RN-18, and RN-19 were selected for BSA based on polymorphism and good amplification.

4.5.1.8. Screening with RY series

Sixteen primers RY-01, RY-03, RY-04, RY-06, RY-07, RY-08, RY-10, RY-11, RY-12, RY-14, RY-15, RY-16, RY-17, RY-18, RY-19 and RY-20 in this series were screened. The amplification pattern observed for different primers in RY series is given in Table 11 and Plate 8b, 10b, 9a, 9a, 10a, 10b, 9a, 8b, 10b, 8b, 10a, 10a, 8b, 10a, 10a and 10b, respectively.

The mean number of bands in this series varied from 0 to 9. The primers which had given polymorphism are RY-01, RY-11, and RY-14. The primer RY-01 has given average 5.5 amplicons in resistant and susceptible parent. One polymorphic band of 0.9 kb was present resistant parent. The primer RY-11 has also given average 5.5 bands in resistant and susceptible parent. It has produced one polymorphic band of 0.4 kb in resistant parent. The primer RY-14 has also given an average polymorphic bands in resistant and susceptible parent. It has produced one polymorphic band of 1.3 kb in resistant parent. The primers RY-01, RY-11, and RY-14 were selected for BSA based on polymorphism and good amplification.

4.5.1.9. Screening with S series

Four primers S-02, S-05, S-15 and S-17 in this series were screened. The amplification pattern observed for S series is given in Table 12 and Plate 7b. The mean number of bands in this series varied from 4 to 9.

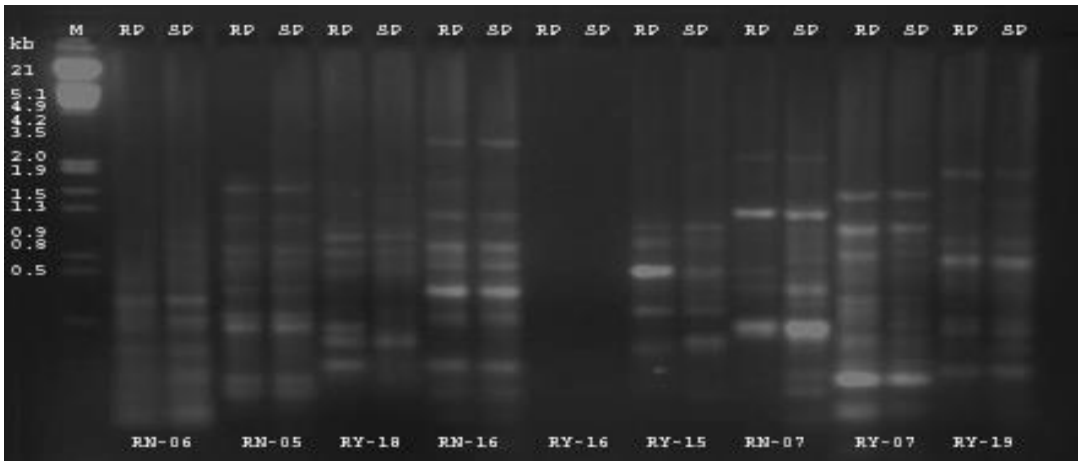
Table 11. Amplification patterns of brinjal genotypes with RY series primers

Sl. No.	Name of Primers	No. of bands		Mean	No. and molecular weight of polymorphic bands		Amplification pattern
		RP	SP			Kb.	
1	RY-01	6	5	5.5	1	0.9	Medium
2	RY-03	5	5	5	0		Medium
3	RY-04	3	3	3	0		Poor
4	RY-06	1	1	1	0		Poor
5	RY-07	5	9	7	0		Good
6	RY-08	0	0	0	0		No amplification
7	RY-10	1	1	1	0		Poor
8	RY-11	6	5	5.5	1	1.3	Medium
9	RY-12	0	0	0	0		No amplification
10	RY-14	6	5	5.5	1	1.3, 0.9	Medium
11	RY-15	5	5	5	0		Medium
12	RY-16	0	0	0	0		No amplification
13	RY-17	2	1	1.5	0		Poor
14	RY-18	6	4	5	2		Medium
15	RY-19	6	7	6.5	0		Good
16	RY-20	5	5	5	0		Medium

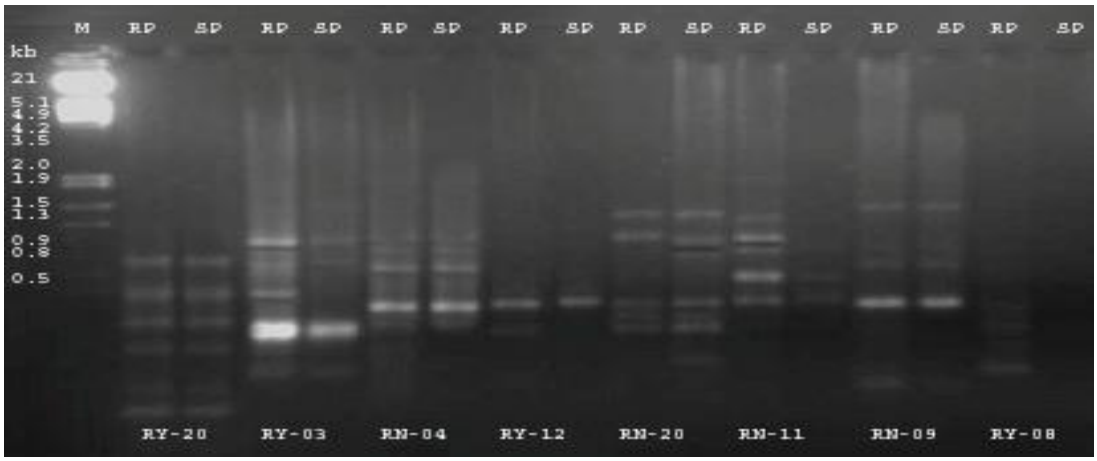
Table 12. Amplification patterns of brinjal genotypes with S series primers

Sl. No.	Name of Primers	No. of bands		Mean	No. and molecular weight of polymorphic bands		Amplification pattern
		RP	SP			Kb.	
1	S-02	5	7	6	0		Good
2	S-05	10	8	9	0		Good
3	S-15	4	4	4	0		Medium
4	S-17	7	7	7	0		Good

RP: Resistant parent SP: Susceptible parent



a)



b)

M: Marker λ DNA / *EcoRI/ HindII*

RP: Resistant parent, SP: Susceptible parent

a) RN-06, RN-05, RY-18, RN-16, RY-16, RY-15, RN-07, RY-07 and RY-19

b) RY-20, RY-03, RN-04, RY-12, RN-20, RN-11, RN-09 and RY-08

Plate 10. Screening of primers of RN and RY series with resistant and susceptible parents.

4.5.1.10. Screening with OPAH-06

The amplification pattern observed for primer OPAH-06 is given in Table 9 and Plate 8a. This primer has given 3 bands in resistant parent and 2 bands in susceptible parent. It has given polymorphic band of 0.9 kb in resistant parent. On repetition, this primer did not amplify 0.9 kb polymorphic band.

4.5.1.11. Screening with OPP-14

The amplification pattern observed for OPP-14 is given in Table 9 and Plate 9b. This primer has given five and four amplicons in resistant and susceptible parent respectively. It has given a polymorphic band of 0.470 kb in resistant parent. This band persisted on repetition. The primer OPP-14 was selected for BSA based on polymorphism, consistency in amplification pattern.

4.5.1.12. Screening with reported wilt specific primers

4.5.1.12.1. Brinjal

Eight primers OPH-02, OPL-04, OPM-01, A-02, PUC-101, GLE-11, SC-10:04 and OPAH-09 which were reported as wilt specific in crop brinjal were screened for polymorphism between resistant and susceptible genotype. The amplification pattern observed is given in Table 13 and Plate 4a, 4b, 4b, 4b, 11a, 4a, 11a, and 7b, respectively. None has produced polymorphism between resistant and susceptible parent. The average amplicons in resistant and susceptible genotype ranged from 3 to 9.5.

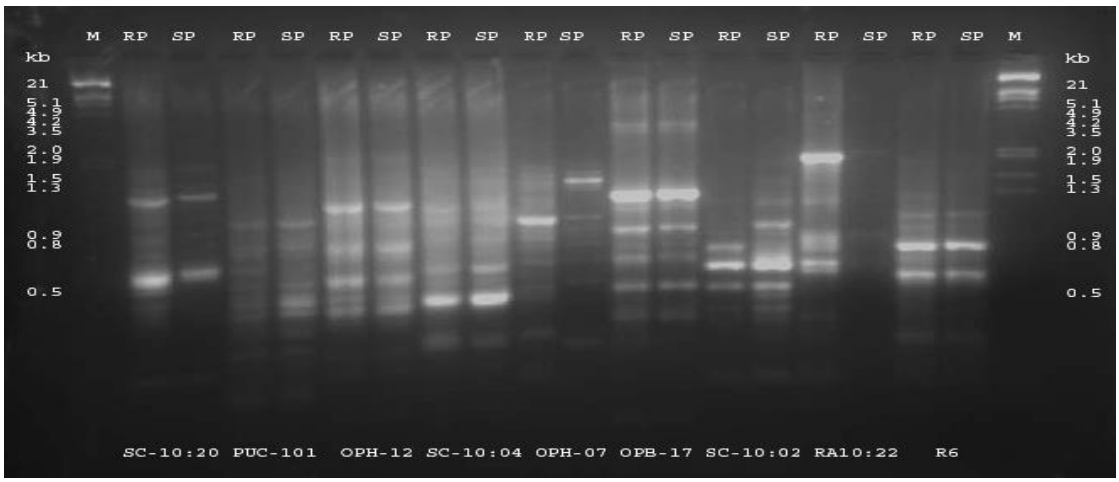
4.5.1.12.2. Tomato

Nine primers AG-08, SC-10:02, RA-10:22, RA-12:13, RA-12:29, RA-12:38, RA-12:41, RA-12:74 and RA-12:84 which were reported as wilt specific to tomato were screened for polymorphism between resistant and susceptible genotypes. Mean number of amplicons by these primers ranged from 0 to 8.5. The

Table 13. Amplification patterns of brinjal genotypes with bacterial wilt specific primers

Sl. No.	Name of Primers	No. of bands		Mean	No. and molecular weight of polymorphic bands		Amplification pattern
		RP	SP			Kb.	
1	A-02	9	9	9	0		Good
2	AG-08	6	6	6	0		Good
3	GLE-11	5	6	5.5	0		Medium
4	OPAG-01	5	5	5	0		Medium
5	OPAH-09	5	6	5.5	0		Medium
6	OPB-17	6	6	6	0		Good
7	OPG-03	6	6	6	0		Good
8	OPG-16	0	0	0	0		No amplification
9	OPH-06	6	6	6	0		Medium
10	OPH-02	10	10	10	0		Good
11	OPH-07	7	7	7	0		Good
12	OPH-12	5	5	5	0		Medium
13	OPL-04	9	9	9	0		Good
14	OPM-01	5	5	5	0		Good
15	PUC-101	7	7	7	0		Good
16	R-06	6	6	6	0		Good
17	R-10	0	0	0	0		No amplification
18	RF	3	3	3	0		Poor
19	RA-10:22	6	6	6	0		Good
20	RA-12:13	4	4	4	0		Medium
21	RA-12:29	4	4	4	0		Medium
22	RA-12:38	3	3	3	0		Poor
23	RA-12:41	7	7	7	0		Good
24	RA 12:74	4	2	3	0		Medium
25	RA-12:84	0	0	0	0		No amplification
26	SC-10:02	5	5	5	0		Medium
27	SC-10:04	4	4	4	0		Medium
28	SC-10:20	4	4	4	0		Medium
29	WG-44	0	0	0	0		No amplification
30	WG-54	8	8	8	0		Good

RP: Resistant parent SP: Susceptible parent



a)

b)

M: Marker λ DNA / *EcoRI/ HindII*

RP: Resistant parent, SP: Susceptible parent

a) SC-10:20, PUC-101, OPH-12, SC-10:04, OPH-07, OPB-17, SC-10:02, RA-10:22 and R-6

b) OPG-16, OPG-03, RA-12:74, RA-12:38, RA-12:29, RF, OPH-06 and RA-12:13

Plate 11. Screening of primers of SC, PUC, OPH, OPB, RA, R, OPG and RF series with resistant and susceptible parents.

amplification pattern observed is given in Table 13 and Plate 4b, 11a, 11a, 12a, 11b, 12a, 12a, 11b and 5a, respectively. No polymorphism was obtained.

4.5.1.12.3. *Solanum commmersonii*

Solanum commmersonii was reported as resistant to bacterial wilt. Nine primers OPB-17, OPG-03, OPG-16, OPH-06, OPH-07, OPH-12, R-6, R-10 and RF reported as wilt specific for this were screened polymorphism between resistant and susceptible genotype. The amplification pattern observed is given in Table 13 and Plate 11a, 11b, 11b, 11b, 11a, 11a, 11a, 5a and 12a, respectively. None has produced polymorphism between them. Mean number of amplicons by these primers ranged from o to 9.5.

4.5.1.12.4. *Solanum torvum*

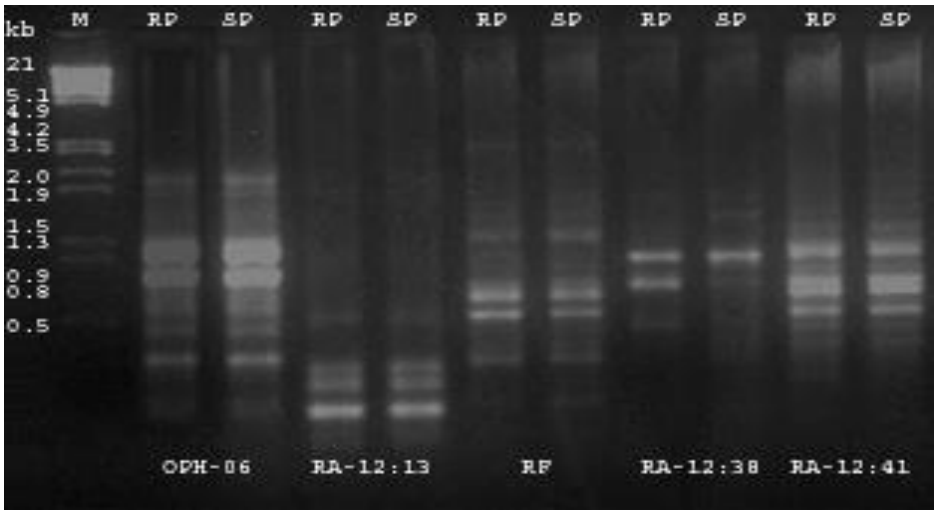
The primers SC-10:04 and SC-10:20 which were reported as wilt specific to *Solanum torvum* was screened polymorphism between resistant and susceptible genotypes. The amplification pattern observed is given in Table 13 and Plate 11a. No polymorphism was obtained.

4.5.1.12.5. Rice

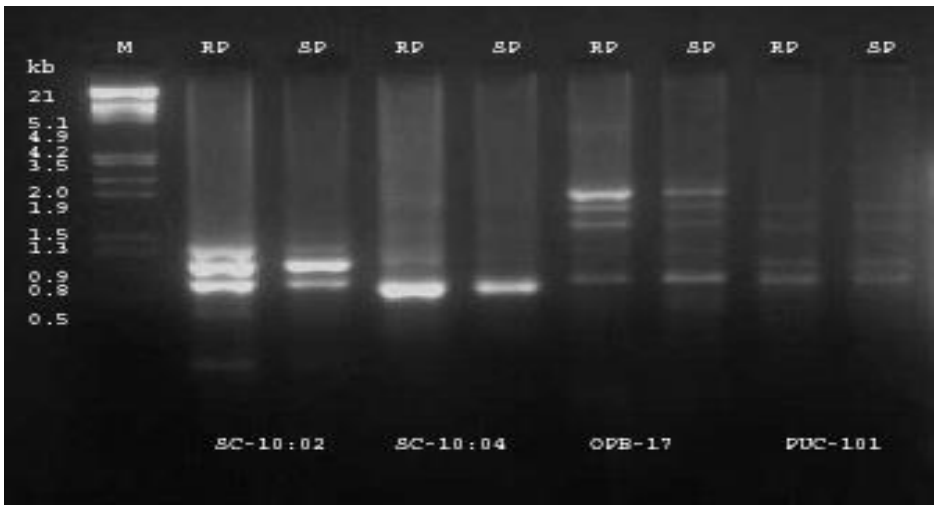
The primers OPAG-01 which was reported as wilt specific to rice was screened for polymorphism between resistant and susceptible genotypes. The amplification pattern observed is given in Table 13 and Plate 4b. No polymorphism was obtained.

4.5.1.12.6. Carnation

The primers WG-44 and WG-54 which was reported as wilt specific to carnation was screened for polymorphism between resistant and susceptible genotypes. The amplification pattern observed is given in Table 13 and Plate 5a and 4b, respectively.



a)



b)

M: Marker λ DNA / *EcoRI/ HindII*

RP: Resistant parent, SP: Susceptible parent

a) OPH-06, RA-12:13, RF, RA-12:38 and RA-12:41

b) SC-10:02, SC-10:04, OPB-17 and PUC-101

Plate 12. Screening of primers of OPH, RA, RF, SC, OPB and PUC series with resistant and susceptible parents.

5.2. Bulked Segregant Analysis (BSA) using selected RAPD primers

BSA was done with seventeen selected primers. The list of primers used for BSA is given in Table 4. The samples for BSA consisted of DNA of resistant parent, susceptible parent, bulked DNA from five resistant F_2 progenies and bulked DNA from five susceptible F_2 progenies, negative control without DNA and marker. Amplification profiles created by each primer were examined for polymorphism between resistant and susceptible genotypes. The results obtained are furnished below. The numbers of bands produced in each genotype by the seventeen primers selected are presented in Table 14.

A-12

Amplification with this primer generated six and five amplicons in resistant parent and susceptible parent respectively of which only one was polymorphic (3kb). The molecular weight of the amplicons ranged between 4.2 kb and 0.5kb. The amplification pattern observed is given in Table 14 and Plate 13a. The polymorphic 3 kb band was present in resistant parent and not in resistant bulk. This primer was unable to differentiate between the resistant and susceptible genotypes.

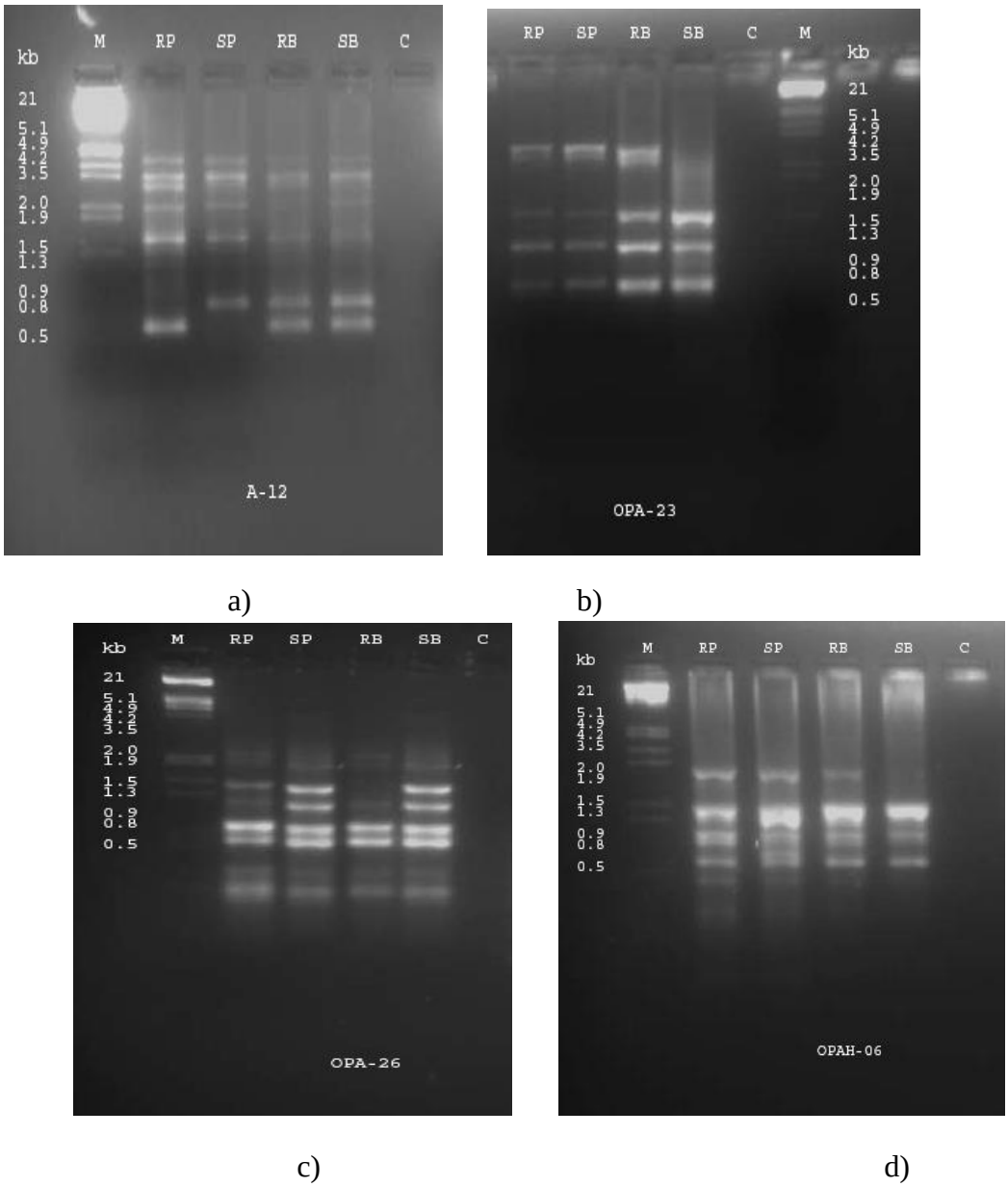
OPA-23

Five amplicons were observed in resistant parent as well as susceptible parent the agarose gel for the DNA amplified with the primer OPA-23. The amplification pattern observed is given in Table 14 and Plate 13b. All bands were monormophic among the genotypes. The molecular weight of the products ranged between 2 kb and 0.5 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

Table 14. Amplification patterns in genotypes of BSA with selected primers

Sl. No.	Name of Primers	No. of bands				No. of polymorphic bands
		RP	SP	RB	SB	
1	A-12	6	5	5	5	0
2	OPA-23	5	5	5	3	0
3	OPA-26	8	8	6	7	0
4	OPA-29	5	5	4	4	0
5	OPA-32	5	6	7	8	0
6	OPAH-6	6	6	4	3	0
7	OPC-09	7	7	8	8	0
8	OPP-14	5	4	5	4	1
9	OPU-03	7	8	7	3	0
10	OPU-09	5	6	2	2	0
11	RN-11	7	8	7	6	0
12	RN-12	4	4	2	2	0
13	RN-18	4	2	2	2	0
14	RN-19	6	5	6	6	0
15	RY-01	6	6	5	4	0
16	RY-11	5	5	5	5	0
17	RY-14	6	6	6	6	0

RP: Resistant Parent, SP:Susceptible Parent, RB: Resistant Bulk, SB: Susceptible Bulk



a) b)

c) d)

M : Marker λDNA /*Hind*III +*Eco*RI, RP: Resistant Parent, SP:Susceptible Parent, RB:Resistant Bulk, SB:Susceptibl Bulk and C:control

Plate 13. BSA with A-12, OPA-23, OPA-26 and OPAH-06 primers

OPA-26

Eight amplicons were observed in resistant parent as well as susceptible parent on the agarose gel for the DNA amplified with the primer OPA-26. The amplification pattern observed is given in Table 14 and Plate 13c. Only one band (1.5 kb) was polymorphic and it was present in resistant parent, resistant bulk and susceptible bulk. The molecular weight of the products ranged between 0.7 kb and 2 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPA-29

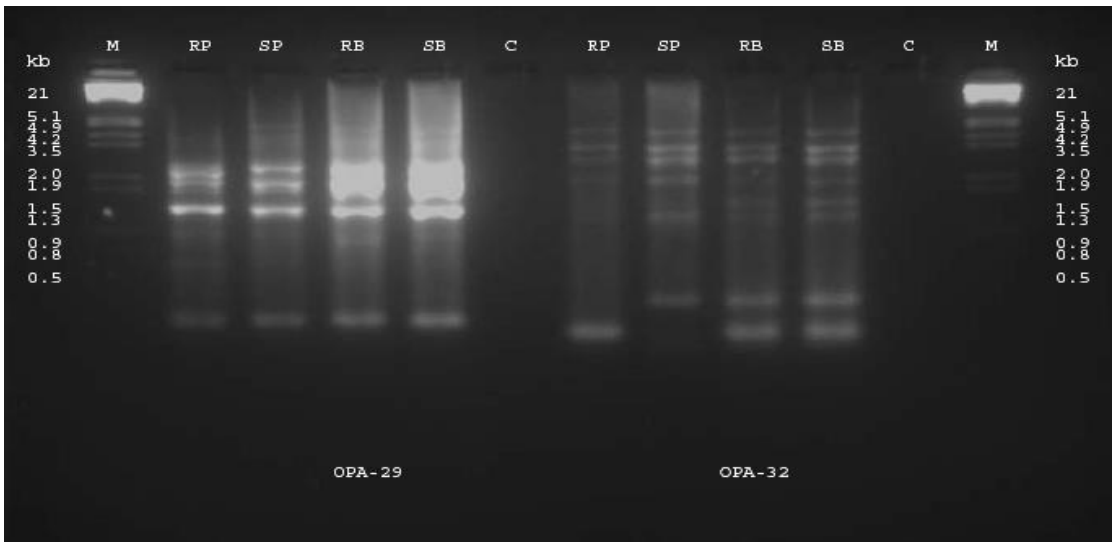
A total of five amplicons were observed in resistant parent and susceptible parent after DNA amplification with the primer OPA-29. The amplification pattern observed is given in Table 14 and Plate 14a. All bands were monomorphic among genotypes. The molecular weight of the products ranged between 2 kb and 0.2 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPA-32

Five and six amplicons were observed in resistant parent and susceptible parent respectively on the agarose gel for the DNA amplified with the primer OPA-32. The amplification pattern observed is given in Table 14 and Plate 14a. One band (0.2 kb) was polymorphic among the genotypes. The molecular weight of the products ranged between 4.2 kb and 0.2 kb. The Polymorphic band (0.2 kb) was present in resistant parent, resistant bulk, and susceptible bulk. This primer was unable to differentiate between the resistant and susceptible genotypes.

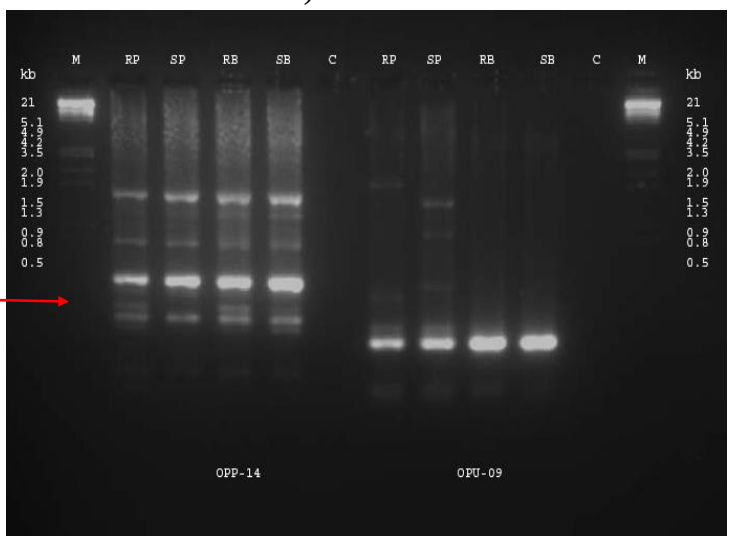
OPAH-6

Six amplicons of each were observed in resistant parent and susceptible parent respectively on the agarose gel for the DNA amplified with the primer OPAH-6. The amplification pattern observed is given in Table 14 and Plate 13d.



a)

470 bp Polymorphic band



b)

M :Marker λDNA /*Hind*III +*Eco*RI , RP: Resistant Parent, SP:Susceptible Parent, RB:Resistant Bulk, SB:Susceptibl Bulk and C:control

Plate 14. BSA with OPA-29, OPA-32, OPP-14 and OPU-09 primers

Only one band (0.5 kb) was polymorphic among the genotypes. This polymorphic 0.5 kb band was seen in the resistant parent but not in resistant bulk. So this primer was unable to differentiate between the resistant and susceptible genotypes. The molecular weight of the products ranged between 1.3 kb and 0.5 kb.

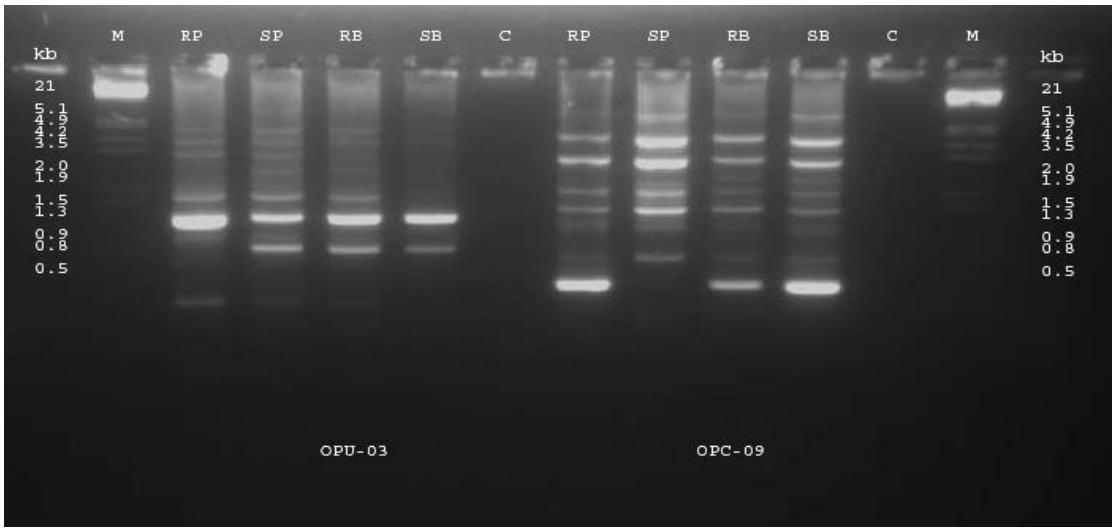
OPC-09

Seven each amplicons were observed in resistant parent and susceptible parent respectively on the agarose gel for the DNA amplified with the primer OPC-09. The amplification pattern observed is given in Table 14 and Plate 15a. All bands were monomorphic among the genotypes. The molecular weight of the products ranged between 4.2 kb and 0.9kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

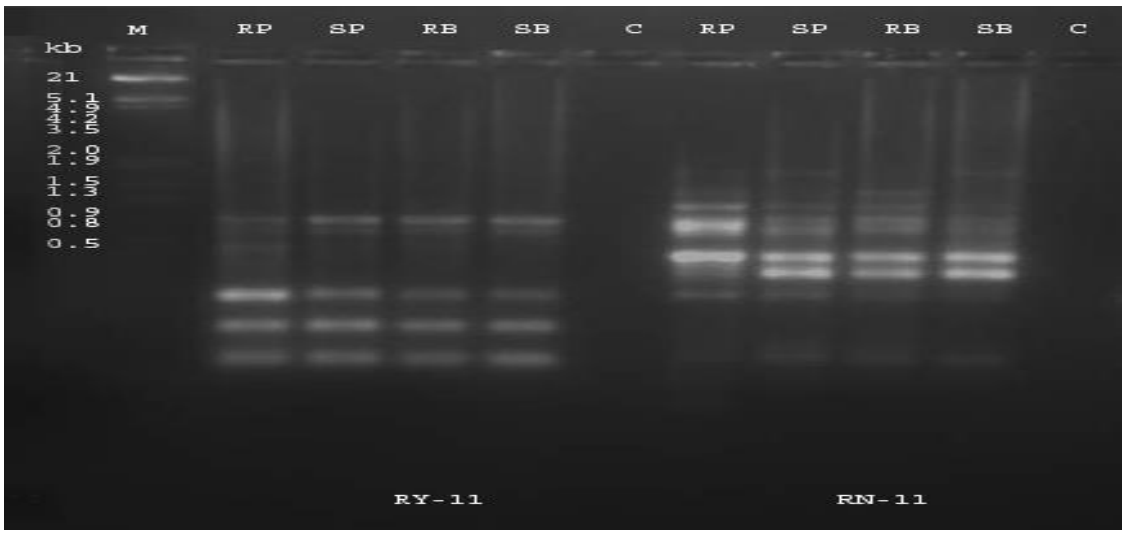
OPP-14

Five and four amplicons were observed in resistant parent and susceptible parent respectively on the agarose gel for the DNA amplified with the primer OPP-14. The amplification pattern generated given in Table 14 and Plate 14b.Three bands were monomorphic and the only one was polymorphic (470 bp) among the genotypes. The molecular weight of the products ranged between 1.3 kb and 0.3 kb. One distinct band of molecular weight (470 bp) was seen in the resistant parent and resistant bulk to bacterial wilt. This primer was able to differentiate between the resistant and susceptible genotypes.

Out of the seventeen selected primers screened for polymorphism among resistant parent, resistant bulk, susceptible parent and susceptible bulk, only OPP-14 has distinguished the individuals which are resistant and susceptible to bacterial wilt disease. This primer was selected for co-segregation analysis of F_2 susceptible and resistant progenies.



a)



b)

M :Marker λDNA /*Hind*III +*Eco*RI , RP: Resistant Parent, SP:Susceptible Parent, RB:Resistant Bulk, SB:Susceptibl Bulk and C:control

Plate 15. BSA with OPU-03, OPC-09, RY-11 and RN-11 primers

OPU-03

Seven and eight amplicons were observed in resistant parent and susceptible parent respectively on the agarose gel for the DNA amplified with the primer OPU-03. The amplification pattern observed is given in Table 14 and Plate 15a. There was no polymorphism among the genotypes. The molecular weight of the products ranged between 4.2 kb and 0.7 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPU-09

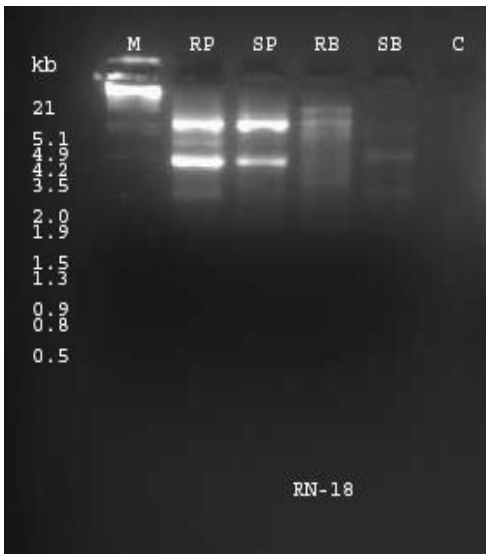
Amplification with this primer generated five and six amplicons in resistant parent and susceptible parent respectively of which one was polymorphic (0.5 kb). This band was present resistant parent only. The molecular weight of the amplicons ranged between 2 kb and 0.1 kb. The amplification pattern observed is given in Table 14 and Plate 14b. This primer was unable to differentiate between the resistant and susceptible genotypes.

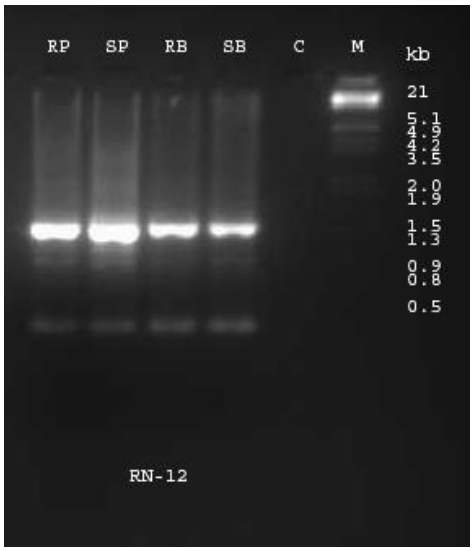
RN-11

Seven and eight amplicons were observed in resistant parent and susceptible parent respectively on the agarose gel for the DNA amplified with the primer RN-11. The amplification pattern observed is given in Table 14 and Plate 15b. All bands were monomorphic among the genotypes. The molecular weight of these markers ranged between 1.9 kb and 0.8 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

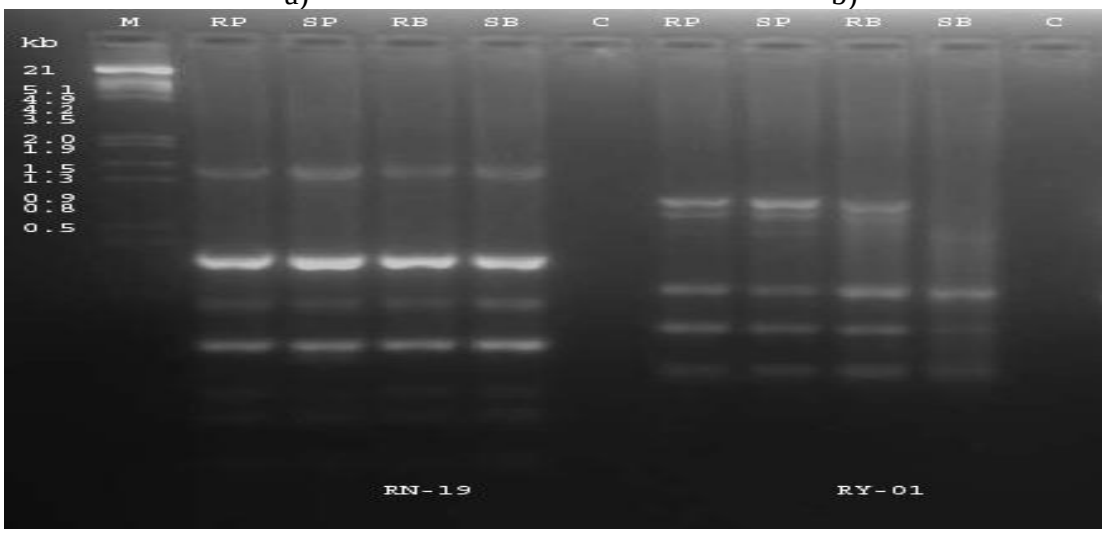
RN-12

Four each amplicons were observed in resistant parent and susceptible parent on the agarose gel for the DNA amplified with the primer RN-12. The amplification pattern observed is given in Table 14 and Plate 16b. All bands were monomorphic among the genotypes. The molecular weight of these markers





a) b)



c)

M : Marker λDNA /*Hind*III +*Eco*RI , RP: Resistant Parent, SP:Susceptible Parent, RB:Resistant Bulk, SB:Susceptibl Bulk and C:control

Plate 16. BSA with RN-18, RN-12, RN-19 and RY-01 primers

ranged between 1.5 kb and 0.5 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

RN-18

Amplification with this primer generated four and two amplicons in resistant parent and susceptible parent respectively of which one was polymorphic (3.5 kb). This band was present only in resistant parent. This primer was unable to differentiate between the resistant and susceptible genotypes. The molecular weight of the amplicon ranged between 1.5 kb and 0.8 kb. The amplification pattern observed is given in Table 14 and Plate 16a.

RN-19

Six and five amplicons were observed in resistant parent and susceptible parent respectively on the agarose gel for the DNA amplified with the primer RN-19. The amplification pattern observed is given in Table 14 and Plate 17b. Only one was polymorphic (0.3 kb). Polymorphic band (0.3 kb) was present in resistant parent, resistant bulk, and susceptible bulk. This primer was unable to differentiate between the resistant and susceptible genotypes. The molecular weight of the products ranged between 1.5 kb and 0.2 kb.

RY-01

Amplification with this primer generated six each amplicons in resistant parent and susceptible parent of which one was polymorphic (0.9 kb). The amplification pattern observed is given in Table 14 and Plate 16c. The molecular weight of the amplicons ranged between 1.2 kb and 0.5 kb. The polymorphic 0.9 kb band was present in resistant parent, resistant bulk, and susceptible parent but was absent in susceptible bulk. This primer was unable to differentiate between the resistant and susceptible genotypes.

RY-11

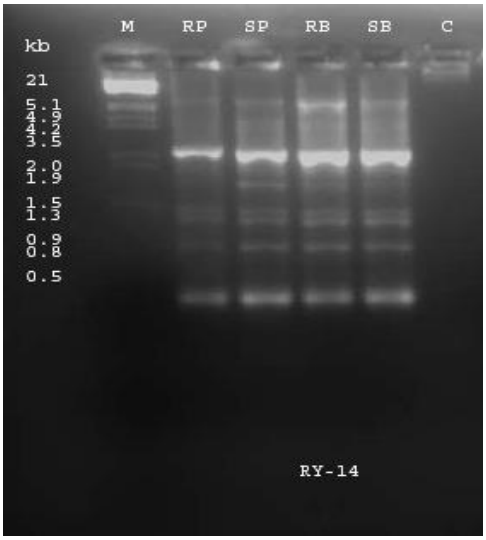
Five each amplicons were observed in parent and susceptible parent on the agarose gel for the DNA amplified with the primer RY-11. The amplification pattern observed is given in Table 14 and Plate 15b. The molecular weight of the products ranged between 1.3 kb and 0.1 kb. Among them one band (0.5 kb) was polymorphic and was present in resistant parent only. It was absent in susceptible parent, resistant bulk, and susceptible bulk. This primer was unable to differentiate between the resistant and susceptible genotypes.

RY-14

A total of six each amplicons were observed in DNA amplification with the primer RY-14 resistant parent and susceptible parent. The amplicons produced with this primer were almost monomorphic for the selected genotypes. The amplification pattern observed is given in Table 14 and Plate 17. The molecular weight of the bands varied from 1.3 kb to 0.5 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

4.5.3. Co-segregation analysis

Co-segregation analysis was carried out with resistant parent, susceptible parent, individuals of resistant and susceptible bulk with selected primer OPP-14. The result is presented in Plate 18. The band of 470 bp was present in resistant parent, resistant bulk, 5 resistant F_2's and three susceptible F_2's. It was absent in susceptible parent and two susceptible F_2's.



M : λDNA /*Hind*III +*Eco*RI , RP: Resistant Parent, SP:Susceptible Parent, RB:Resistant Bulk, SB:Susceptibl Bulk and C:control

Plate 17. BSA with RY-14 primer

470 bp Polymorphic band

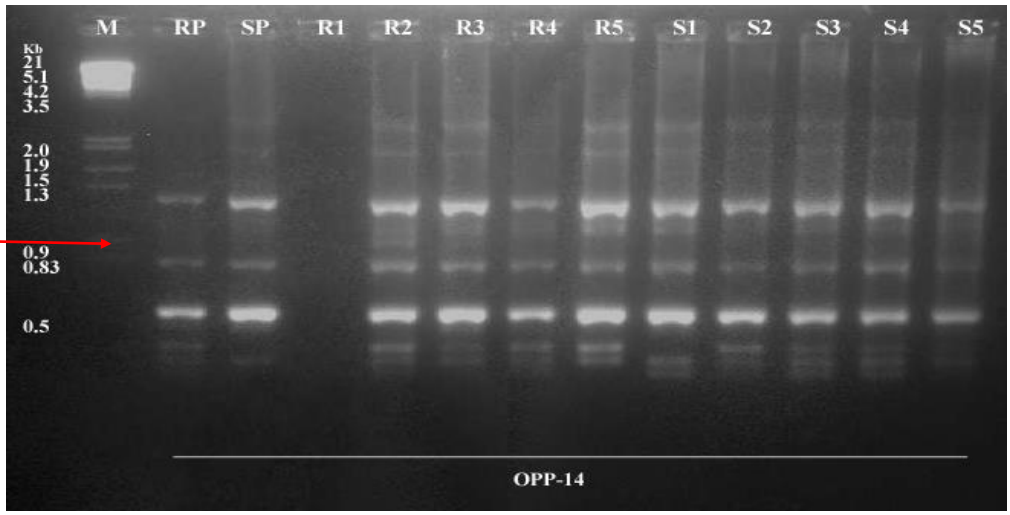


Plate 18. Co-segregation analysis in brinjal genotype with OPP-14 primer

M : Marker λDNA /*Hind*III +*Eco*RI , RP: Resistant Parent, SP:Susceptible Parent, R1 to R5 resistant F_2s and S1 to S5 susceptible F_2s

4.6. Molecular Cloning

The distinct band of 470 bp amplified by OPP-14 primer in resistant parent, resistant bulk and five resistant F_2 progenies was eluted from resistant parent for cloning.

4.6.1. Detection of trait specific marker

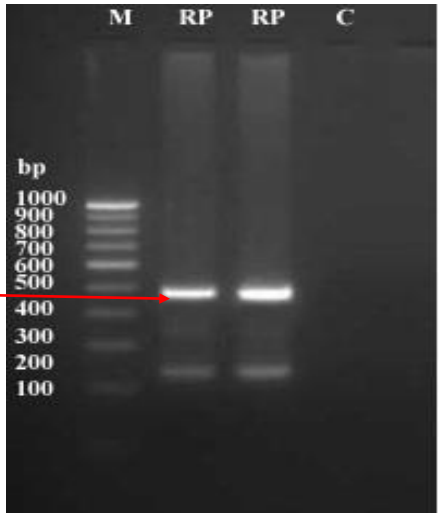
In order to elute the DNA of the specific band of 470 bp, four RAPD reactions with the OPP-14 primer and template DNA from resistant parent were setup. The RAPD products were resolved on 1.4 per cent agarose gel (Plate 19a) and the band of interest was cut from each lane, lifted, and pooled. The DNA of the specific amplicon was eluted as mentioned in section 3.2.3.7.2. The specific band of size 470 bp was absent in control (genomic DNA susceptible parent).

The eluted product (2 µl) was checked on 1 per cent agarose gel and band of the same molecular weight was observed. The bands obtained after elution were intact and of good quality (Plate 19b). The quantity of DNA was 28ng/ul and UV (A_{260}/ A_{280}) absorbance ratio was 1.88 as per the Nanodrop. This eluted product was stored -20^0C and used for cloning and sequencing study.

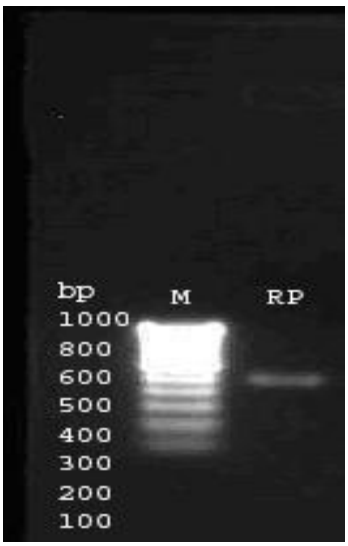
4.7. Transformation of *E.coli*

4.7.1. Preparation of competent cells:

Competent cells were prepared from *E. coli* JM 109 strain (Plate 20) as per the procedure described in Section 3.2.3.8.1. Sufficient multiplication of cells was obtained by culturing cells at 37°C at 160 rpm for 3.5 hours in LB broth. Treating the cells with 0.1 M $CaCl_2$ at low temperature *viz,* 0°C to 4°C might has imparted the competency for intake of foreign DNA.



a)



b)

Plate 19. a) Gel elution b) Eluted product on agarose

M : Marker 100 bp ladder, RP: Resistant Parent and C:control

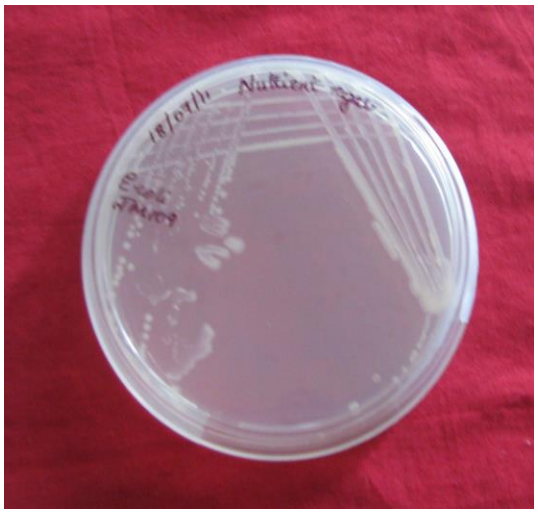


Plate 20. *E. coli* JM-109 strain

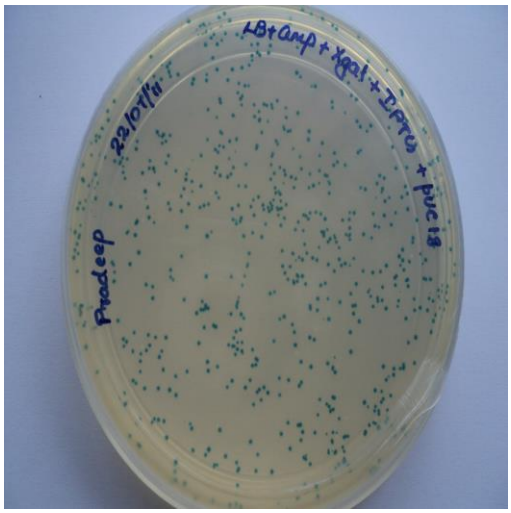


Plate 21. Confirmation of competency

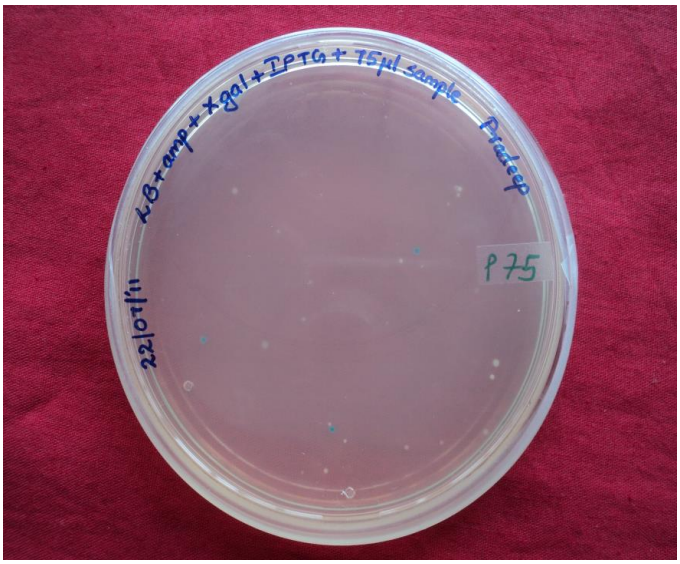


Plate 22. Blue and white colonies after cloning and transformation

4.7.2. Screening of competent cells:

Large number of blue colonies were obtained when the competent cells of *E.coli* after transformation using pUC18 vector (Fig.1) containing ampicillin resistance gene and *LacZ* gene was cultured in LBA medium containing X-gal and IPTG. The colonies showed luxuriant growth, with no other contamination in LBA ampicillin plates (Plate 21) which indicated a high degree of transformation efficiency. Thus the competent cells prepared were found ideal for transformation and further cloning purposes.

4.8. Cloning of eluted DNA with pGMT vector

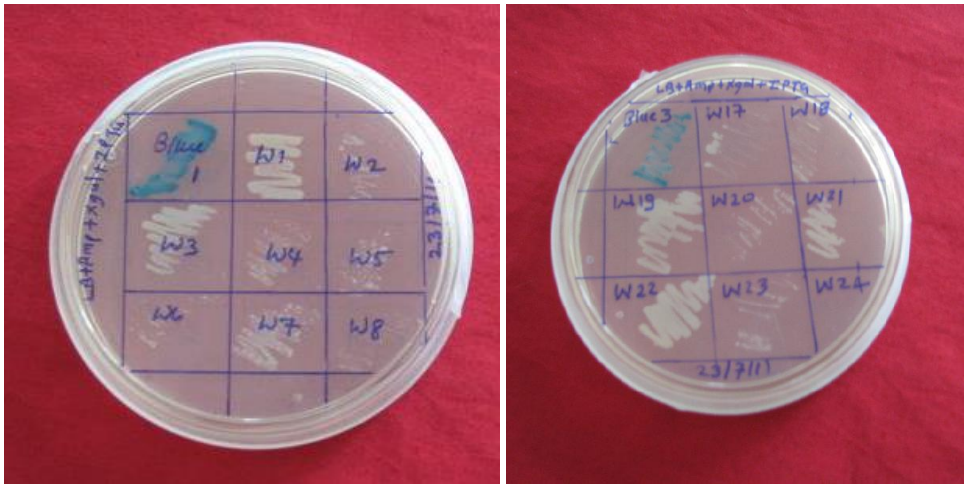
Cloning of eluted DNA in pGEMT vector system (Fig. 2) was done as described in section no. 3.2.3.9. and the product were stored at 4^0C for further used.

4.8.1 Cloning of ligated DNA in competent cells

The cloning of ligated DNA in competent cells was done as described in section 3.2.3.9.2. The recombinant clones were selected based on blue white screening. The white colonies represent transformed and blue colonies represent non-transformed ones (Plate 22). Few white, blue and positive control colonies were carefully transferred to the prepared LB/ampicillin (50mg/l) grid plates layered with IPTG (6µl) and X-gal (60µl) and the colonies were numbered in each plate. The plates were incubated at 37°C overnight and further stored at 4°C (Plate 23). Each colony was given an identity number.

4.9. Confirmation of the DNA insert by colony PCR

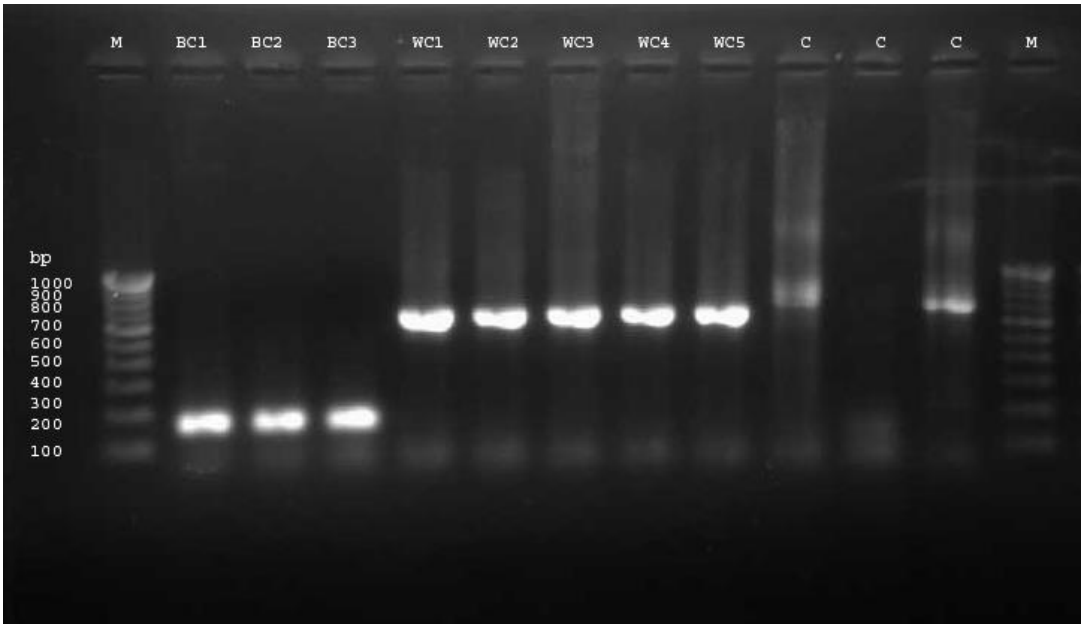
Confirmation of insert was carried out by performing colony PCR of recombinant and non-recombinant plasmid DNA using T7 forward and SP6 reverse universal primers as described in section 3.2.3.10. The amplicons from white colonies produced 700 bp bands while blue colonies 200 bp base pair bands



a) b)

Plate 23. Subcultured blue and white colonies in the plates

M:



BC: Blue colonies

WC: White colonies

C: Control

Plate 24. Confirmation of DNA insert by colony PCR

(Plate 24). The positive control for white colonies produced 750 bp bands and it was 175 bp in blue colonies.

4.10. Sequencing of DNA clone

Recombinant clone *W-3* was sequenced using T7 and ABI Prism sequence analyzer and data obtained is presented in Fig 3.

4.11. *In Silico* analysis of sequences

4.11.1. Vector screening

The sequence obtained after automated sequencing was subjected to Vector screening using the VecScreen tool in NCBI to remove vector regions from the clone. In *W-3* clone the region starting from 408 to 402 bases showed similarity with pGEM-T Easy Vector System sequence (Fig. 4), and it was deleted from the original DNA sequence by BioEdit Biological sequence alignment editor tool. The sequence of 408 bases obtained after vector screening was named as "*Sol-3*" and is presented in Fig 5.

4.11.2. Nucleic acid and Protein sequence analysis

The following BLAST programes were used:

3. **Nucleotide- nucleotide BLAST (Blastn)**

The nucleotide sequence of "*Sol-3*" was compared with the sequences available in nucleotide database using Blastn tool (www.ncbi.nlm. nih.gov/blast/; Altschul *et al.*, 1997) provided by NCBI. The result is shown in shown in Fig 6. Blastn search showed homology with AC237888 *Solanum tuberosum* clone RH183L22 published sequences present in public database NCBI. It has shown 77 per cent identity to AC237888 *Solanum tuberosum* clone RH183L22.

>W3_T7

GCCGAACATCAAAGGTCGGGTGGCCTAGCCCAAGACATAAAGATTCCT
ACTTTAAAGTGGGATGAAATTAATATGGATATTTTGGTTAGGTTGCCAA
AGACTAGGAAGGGTTTTGATTCTATTTGGATTATGGTTGATATAATGAC
AAAATCTTTCATTTTCGACCGGTTAAGACTATATATGGGGTTGAAGACT
ATGCTAGGTTGTACATTTATGACCTGGTGAGGTTGCATGGGATGTCTTTA
TTCATTATATTGGATCGCGGTGCTCAATTTACTTCACATTTTTGGAGGTC
CTTTCAATGGGGTCTTGGTACAAAATTTAAGAATAGTACTGCGTTCTGA
CATATCCCCTCAGAACTTCCCCAAAGAGCAACCATGTGTGAATAAATTT
TGAGCTAATGCGCTTGCTCCCACAATGAAATCTTCCCGTGTGATTGTGTA
TCCAAAATGCGTCAAAAATTCCAAGCCTAACCGAACTGATGAGAGTACG
TTTCGATTTCTGACTGTGTTAGCCTGAGAAGTGCTTGTCCCAACCTTGTT
TCTGAGCATGAACGCCCGCAAGCCAACATGTTAGTTGAAACATCAGGGC
GATTATCATATGATATCAAAACGCTCTGAGCTGCTCGTTCGGCTATGGC
GTAGCCTAGTCCGTAAGCAGGACTTTTCTGGTCTCGCAAGGTTTCTTCAA
TCTGCATTCGCTTCGAATAGATATTCACTA

Fig 3. Nucleotide sequence of brinjal *"W-3"* amplicon

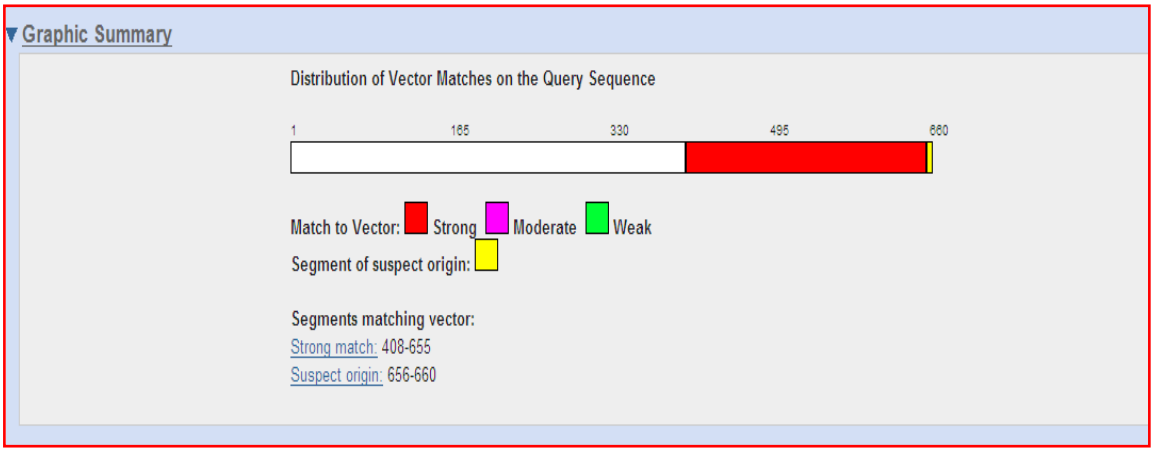
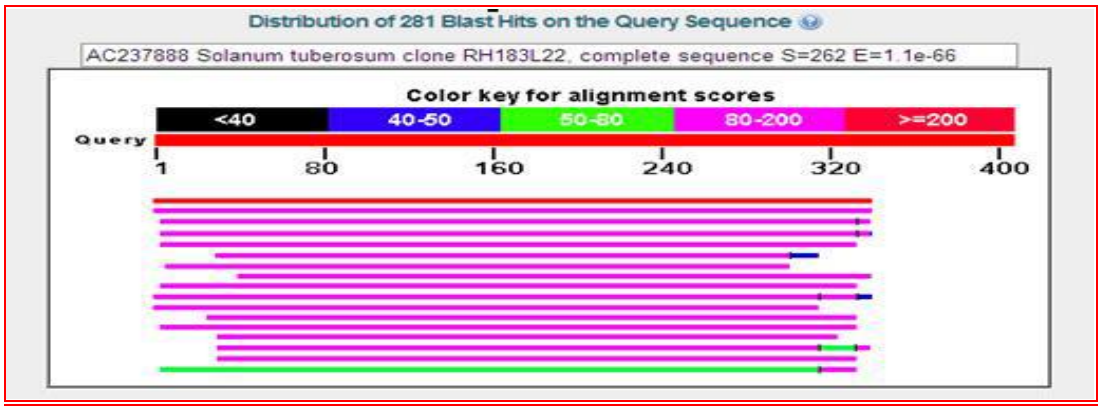


Fig 4. VecScreen output of brinjal *"W-3"*

>

GCCGAACATCAAAGGTCGGGTGGCCTAGCCCAAGACATAAAGATTCCT
ACTTTAAAGTGGGATGAAATTAATATGGATATTTTGGTTAGGTTGCCAA
AGACTAGGAAGGGTTTTGATTCTATTTGGATTATGGTTGATATAATGAC
AAAATCTTTCATTTTCGACCGGTTAAGACTATATATGGGGTTGAAGACT
ATGCTAGGTTGTACATTTATGACCTGGTGAGGTTGCATGGGATGTCTTTA
TTCATTATATTGGATCGCGGTGCTCAATTTACTTCACATTTTTGGAGGTC
CTTTCAATGGGGTCTTGGTACAAAATTTAAGAATAGTACTGCGTTCTGA
CATATCCCCTCAGAACTTCCCCAAAGAGCAACCATGTGTGAATAAATTT
TGAGCTAATGCGCTTC

Fig 5. Brinjal *"Sol-3"* Nucleotide sequence after deleting vector sequence



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AC237888.1	Solanum tuberosum clone RH183L22, complete sequence	262	339	83%	1e-66	77%	
AC240345.1	Solanum tuberosum chromosome unknown clone RHPOTKEY012A20, complete	150	282	83%	5e-33	70%	
AC244941.2	Solanum lycopersicum strain Heinz 1706 chromosome 10 clone sle-19f15 map	147	399	82%	6e-32	70%	
AC244036.2	Solanum lycopersicum strain Heinz 1706 chromosome 10 clone hba-11b4 map	147	385	82%	6e-32	70%	
AP009271.1	Solanum lycopersicum genomic DNA, chromosome 8, clone: C08HBa0074N03,	141	238	81%	3e-30	69%	
AC233394.1	Solanum tuberosum chromosome 1 clone RH051F22, complete sequence	140	261	70%	9e-30	71%	
AP009318.1	Solanum lycopersicum genomic DNA, chromosome 8, clone: C08HBa0022O08,	136	136	72%	1e-28	71%	
AC244074.7	Solanum lycopersicum strain Heinz 1706 chromosome 10 clone hba-7b3 map 1	134	427	73%	4e-28	73%	
AC245329.3	Solanum lycopersicum strain Heinz 1706 chromosome 10 clone sle-44l16 map	131	131	81%	5e-27	68%	
AC245349.3	Solanum lycopersicum strain Heinz 1706 chromosome 10 clone sle-46e12 map	127	435	83%	6e-26	74%	
AC244801.2	Solanum lycopersicum strain Heinz 1706 chromosome 10 clone hba-54n12 map	127	293	77%	6e-26	74%	
AC243015.1	Solanum tuberosum chromosome 11 clone RH009L13, complete sequence	127	127	75%	6e-26	69%	
AP010941.1	Solanum lycopersicum DNA, chromosome 8, clone: C08HBa0205P22, complete	127	510	81%	6e-26	73%	
AC245787.3	Solanum lycopersicum strain Heinz 1706 chromosome 1 clone sle-6a7 map 1,	125	235	72%	2e-25	69%	
AP010933.1	Solanum lycopersicum DNA, chromosome 8, clone: C08SLm0111K07, complete	125	299	75%	2e-25	71%	
AC245980.6	Solanum lycopersicum strain Heinz 1706 chromosome 1 clone sle-45d20 map 1	123	476	74%	7e-25	71%	
AP010799.1	Solanum lycopersicum DNA, chromosome 8, clone: C08HBa0112M02, complete	123	188	81%	7e-25	69%	
AP009546.1	Solanum lycopersicum DNA, chromosome 8, clone: C08HBa0057A12, complete	123	245	73%	7e-25	70%	
AP009409.1	Solanum lycopersicum genomic DNA, chromosome 8, clone: C08HBa0008F16,	123	286	73%	7e-25	70%	
CU062424.8	S.lycopersicum DNA sequence from clone LE_HBa-147F16 on chromosome 4,	123	170	74%	7e-25	69%	
AC244711.7	Solanum lycopersicum strain Heinz 1706 chromosome 10 clone hba-77i11 map	122	177	75%	2e-24	69%	
AC244894.1	Solanum lycopersicum strain Heinz 1706 chromosome 10 clone sle-25b5 map 1	122	236	75%	2e-24	69%	
AC244564.2	Solanum lycopersicum strain Heinz 1706 chromosome 10 clone hba-38n9 map	122	170	81%	2e-24	72%	

Fig 6. Blastn output of brinjal *"Sol-3"* sequence.

2. Protein- protein BLAST (Blastp)

The nucleotide sequence of "*Sol-3*" was translated into amino acids using EMBOSS Transeq tool (http://www.ebi.ac.uk/Tools/emboss/transeq/). The result obtained is given as Fig. 7. After removing the non-coding sequences, the amino acids sequences were subjected homology search for known protein with Blastp. It has showed homology with ABC948993 polyprotein (*Oryza australiesis*) S=76 E=1.6e-14 present in public database NCBI with 76.3 total score (Fig 8).

3. Detection of Open Reading Frame (ORF)

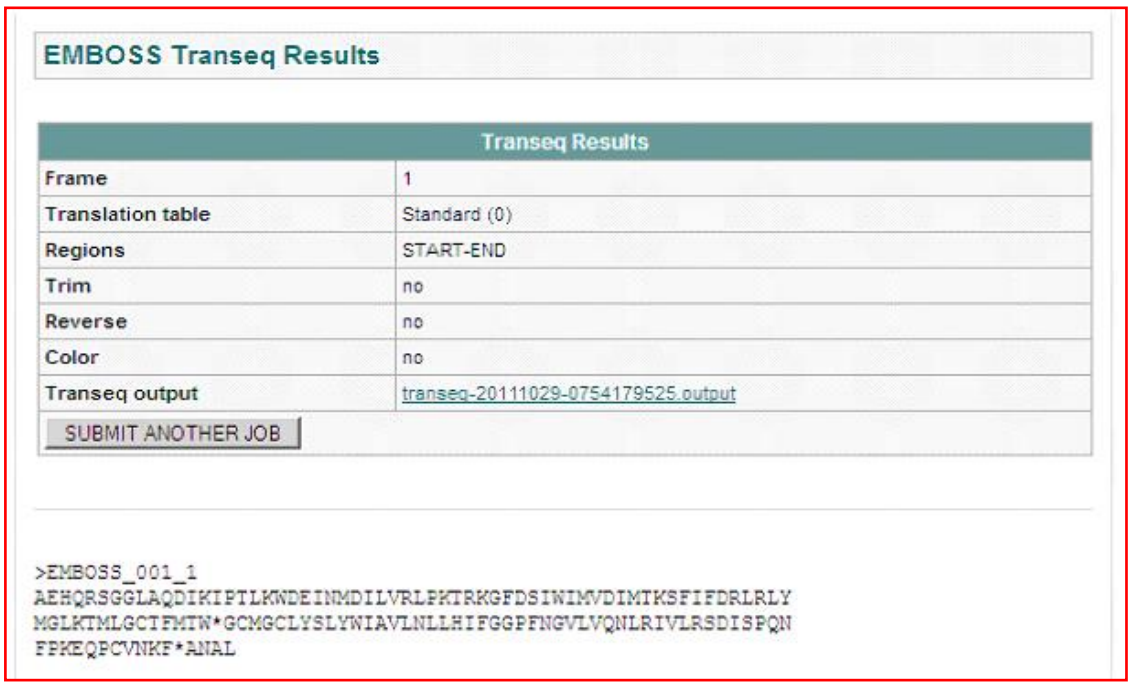
The sequence data of "*Sol-3*" was subjected to ORF finder analysis using NCBI tool ORF finder.

ORF analysis revealed four ORF frame in "*Sol-3"* sequence, three of them located on the plus strands (+1, +1 and +3) and one located on minus (-2). The longest ORF was on +3 frame with a length of 108 bp which revealed significant level of homology with putative retrotransposon protein in *Solanum demissum* with 88% query coverage and 71% of identity published sequences present in public database NCBI. The details are provided in Fig. 9.

4.12. Development of STS marker

4.12.1. STS primer designing

Two Sets of 22 bp length forward and reverse primers was designed from the sequence information of cloned RAPD fragment OPP-14 "*Sol-3*" using Primer3Plus tool is given in fig10. The criteria considered in designing the primer is described in section 3.2.4.1. The sequence data of primers is given in Table 15.



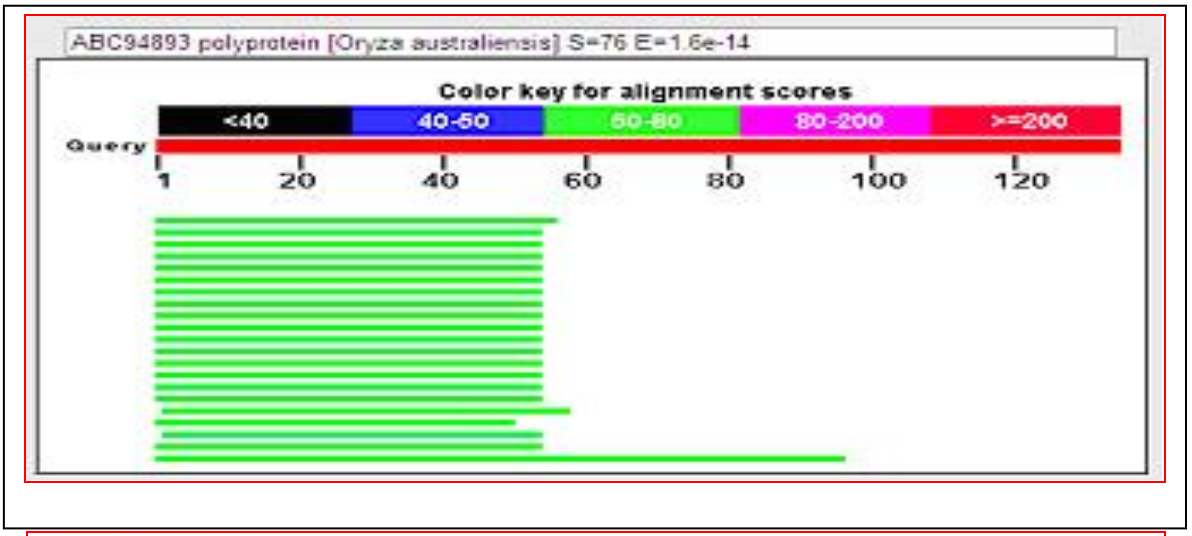
EMBOSS Transeq Results

Transeq Results	
Frame	1
Translation table	Standard (0)
Regions	START-END
Trim	no
Reverse	no
Color	no
Transeq output	transeq-20111029-0754179525.output

SUBMIT ANOTHER JOB

```
>EMBOSS_001_1
AEHQRSGGLAQDIKIPTLKNDEINMDILVRLPKTRKGFDSIWIMVDIMTKSFIFDRLRLY
MGLKTMLGCTFMTW*GCMGCLYSLYWIAVLNLLHIFGGPFNGVLVQNLRIVLRSDISPQN
FPKEQPCVNKF*ANAL
```

Fig 7. EMBOSS Transeq results for amino acid



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Links
CAD39542.3	OSJNBa0057M08.12 [Oryza sativa Japonica Group]	77.0	77.0	41%	9e-15	
ABC94893.1	polyprotein [Oryza australiensis]	76.3	76.3	40%	2e-14	
CAE04199.2	OSJNBa0011E07.8 [Oryza sativa Japonica Group]	75.5	75.5	40%	3e-14	
ABA97430.1	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa Japonica Group	75.5	75.5	40%	3e-14	
AAT81703.1	putative reverse transcriptase, 3'-partial [Oryza sativa Japonica Group]	75.5	75.5	40%	3e-14	
ABF97606.1	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa Japonica Group	75.5	75.5	40%	3e-14	
AAR00610.1	putative reverse transcriptase [Oryza sativa Japonica Group]	75.5	75.5	40%	3e-14	
AAA98111.1	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa Japonica Group	75.5	75.5	40%	3e-14	
ABA93860.1	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa Japonica Group	75.5	75.5	40%	3e-14	
CAH67609.1	OSIGBa0145G11.8 [Oryza sativa Indica Group]	75.5	75.5	40%	3e-14	
AAP53838.1	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa Japonica Group	75.5	75.5	40%	3e-14	
CAD41450.1	OSJNBa0019D11.8 [Oryza sativa Japonica Group]	75.5	75.5	40%	3e-14	
CAD39763.2	OSJNBa0059D20.6 [Oryza sativa Japonica Group]	75.5	75.5	40%	3e-14	
AAM14684.1	Putative polyprotein [Oryza sativa Japonica Group] >gb|AAN04934.1| Putative polyp	75.5	75.5	40%	3e-14	
ABG22100.1	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa Japonica Group	75.5	75.5	40%	4e-14	
ABA94586.1	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa Japonica Group	75.5	75.5	40%	4e-14	
AAT38744.1	Putative gag-pol polyprotein, identical [Solanum demissum]	74.7	74.7	42%	7e-14	
CAH66016.1	OSIGBa0093M15.6 [Oryza sativa Indica Group]	74.3	74.3	37%	8e-14	
AAT38724.1	Putative retrotransposon protein, identical [Solanum demissum]	74.3	74.3	39%	8e-14	
ADD83155.1	putative retrotransposon protein [Phyllostachys edulis]	74.3	74.3	40%	9e-14	
ABF97872.1	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa Japonica Group	74.3	74.3	71%	9e-14	
AAM01060.1	Putative retroelement [Oryza sativa Japonica Group] >gb|AAM74406.1|AC120497_6	73.9	73.9	40%	1e-13	
ABA95216.1	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa Japonica Group	73.6	73.6	40%	1e-13	
CAE02265.2	OSJNBb0049I21.5 [Oryza sativa Japonica Group]	73.6	73.6	40%	1e-13	

Fig 8. Blastp output of brinjal *"Sol-3"* sequence.

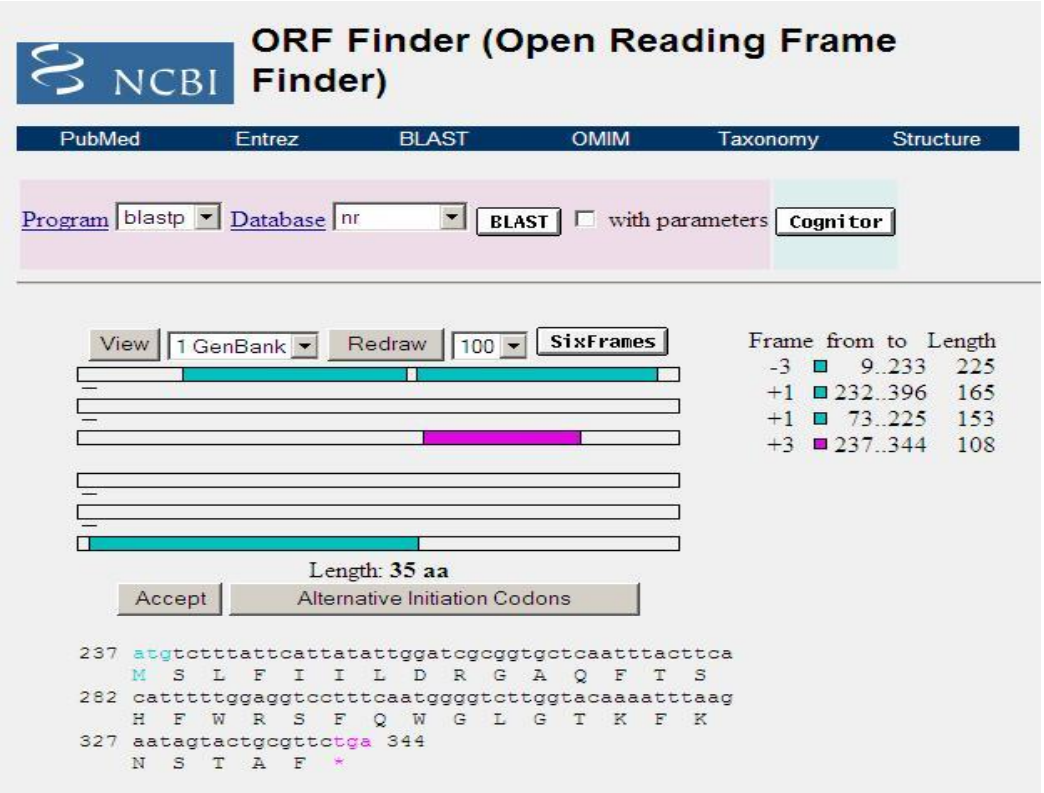


Fig 9. ORF finder output of "*Sol-3*" sequence

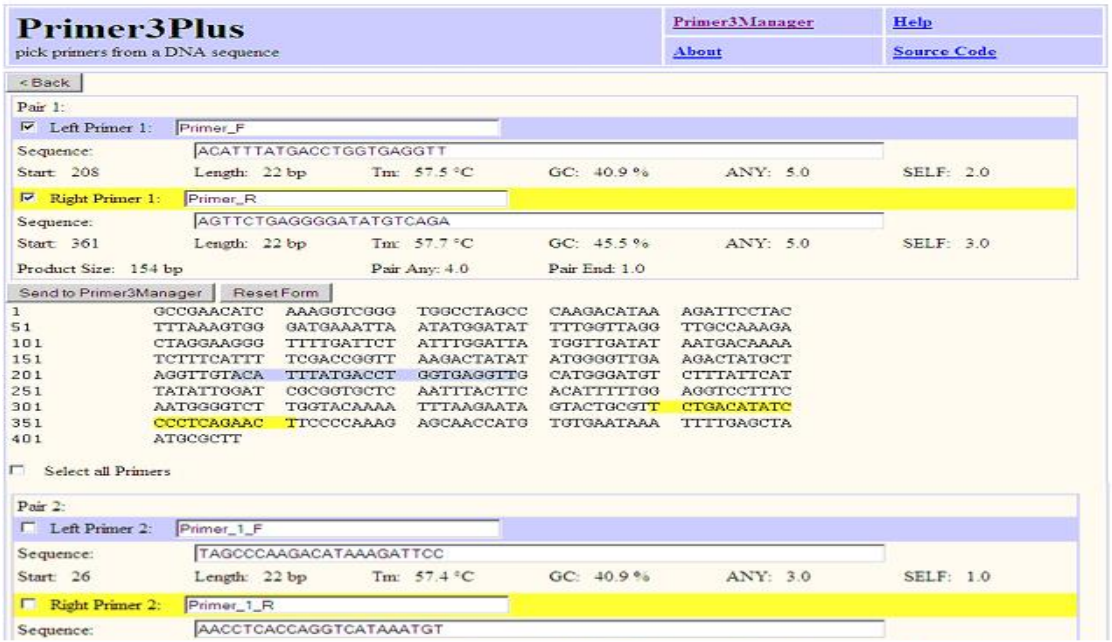


Fig 10. STS Primer designing by Primer3Plus tool

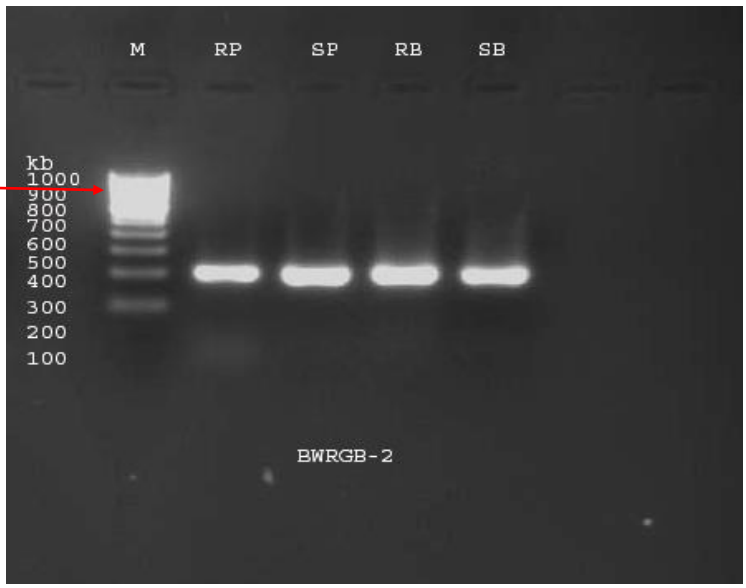
Sl. No	Name of primers	Sequence	No. of Bases
Set -1	BWRGB 1 Forward	5' ACATTTATGACCTGGTGAGGTT 3'	22
	BWRGB 1 Reverse	3' AGTTCTGAGGGGATATGTCAGA 5'	22
Set -2	BWRGB 2 Forward	5' TAGCCCAAGACATAAAGATTCC 3'	22
	BWRGB 2 Reverse	3' AACCTCACCAGGTCATAAATGT 5'	22

Table 15. Details of STS primer sets

4.12.2. PCR analysis of STS primers

The DNA from resistant parent (*Solanum melongena* var. *insanum* I.C. number 421463), susceptible parent (Pusa Purple Long), resistant bulk, and susceptible bulk (Plate 26) were amplified with STS primer BWRGB-2. A negative control was also kept without template DNA. PCR was done as detailed in section 3.2.4.3. in Mastercycler. The amplified products were electrophorised on 2 per cent agarose gel (Plate 25). STS primers amplified 200bp in all samples tested. The designed STS primers were not able to distinguish between resistant and susceptible genotypes of brinjal to bacterial wilt caused by *Ralstonia solanacearum*.

200 bp amplified band



M:Marker 100 bp ladder, RP: Resistant parent, SP: Susceptible parents RB: Resistant bulk, SB: Susceptible bulk

Plate 25. Amplification of brinjal genotypes with STS primer BWRGB 2

Discussion

5. DISCUSSION

Bacterial wilt caused by the soil-born plant pathogen *Ralstonia solanacearum* (Smith 1896, Yabuuchi *et al*., 1995) is one of the most devastating bacterial plant diseases of tropical, subtropical and temperate regions of the world. It has an unusually wide host range, over 200 belonging to more than 50 botanical families (Hayward 2000). It is a major threat to the cultivation of economically important crops such as tomato, potato, eggplant and chilli in India (Vanitha *et al.,* 2009) and others like ginger, banana, groundnut etc. The disease incidence is very much influenced by environmental conditions and resistance/susceptibility reaction of the host.

Various conventional management strategies have been developed for controlling bacterial wilt. But the control of the same has proved to be very difficult, due to its broad host range and soil borne nature. Applications of chemical pesticide are not only harmful to human health and the environment, but also not fully effective. The most widely accepted and promising strategy is breeding resistant cultivars. The conventional breeding methods have given excellent results, but they are time consuming and expensive. Moreover, the pathogen populations adapt to resistant cultivars at a faster rate. This necessitates the exploration of more efficient selection and breeding strategies than those currently existing.

Biotechnology offers tools for gene tagging and isolation from resistant sources and introgression into desirable agronomic backgrounds. Wild varieties of cultivated species show varying degrees of resistance to pest and disease. Bacterial wilt resistance has been reported in the following eggplant genotypes *viz, S. sisymbrifolium* Lam. and *S. torvum* Sw.(1) *S. capsicoides* All., (2) *S. sisymbrifolium* Lam. (p. r.), (3) *S. sessiliflorum* Dun., (4) *S. stramonifolium* Jacq., (5) *S. virginianum* L., (6) *S. aethiopicum* gr. *aculeatum*, (7) *S. grandiflorum* Ruiz & Pavon, (8) *S.hispidum* Pers., (9) *S. torvum* Sw., (10) *S. nigrum* L., (11) *S. americanum* Mill., (12) *S. scabrum* Mill (Collonnier *et al*., 2001).

Gopimony (1983) had conducted genetic analysis of bacterial wilt resistance in *Solanum melongena* var. *insanum* (wild variety) by crossing the same with cultivated variety Pusa Purple Giant. He found that F_1 is resistant and susceptible F_2 plants showed resistance at the ratio of 3:1. According to him the bacterial wilt resistance in this wild brinjal is monogenic and dominant. It is easy to tag and clone such R genes and are desirable for genetic manipulations. Molecular markers linked to the gene of interest can complement traditional breeding programs viz., MAS.

The strategies followed to tag major genes include parental and progeny survey with molecular markers coupled with construction of near isogenic line (NIL). Since construction of NIL is time consuming and tedious, major genes can be mapped by another alternative strategy, bulk segregant analysis (Michelmore *et al*., 1991). Molecular markers used for gene tagging is RAPD, SSR, AFLP, SCAR, ISSR and STS.

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 which linked loci might be detected as polymorphic between the bulked samples.

The present study aimed at Tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* by molecular markers with RAPD and STS. Resistant variety *Solanum melongena* var. *insanum*, susceptible variety Pusa Purple Long and segregating F_2 population of cross *Solanum melongena* var. insanum x Pusa Purple Long served as source of DNA for molecular marker analysis. The methodology involved was bulk segregant analysis. The technical programme involved the following aspects:

i. Development of segregating F_2 generation for the trait under consideration.
ii. Phenotyping of genotypes for bacterial wilt resistance.
iii. Molecular characterization of brinjal genotypes with RAPD marker for getting polymorphism between resistant and susceptible genotypes.
iv. Conversion of RAPD marker into STS marker for tagging of bacterial wilt resistance gene.

The results obtained on various aspects are discussed here under.

5.1. Plant materials for the study

The genotypes selected were for Pusa Purple Long susceptible and *Solanum melongena* var. *insanum* I. C. number 421463 resistant to bacterial wilt were observed for morphological characters given in the Table 1. The parents showed high contrast with respect to fruit length, fruit colour, fruit shape and spiny nature. The fruit length was 27.5 cm for Pusa Purple Long and 5 cm for *Solanum melongena* var. *insanum*.

5.2. Development of segregating F_2 generation for trait under consideration

Controlled crosses were made using the resistant variety *Solanum melongena* var. *insanum* I. C. number 421463 as the female parent (Plate 1a) and susceptible variety Pusa Purple Long as the male parent (Plate 1b), and F_1 plants were generated (Plate 1c). In crossing to raise F_1 generation the fruit setting was only 11 per cent. There is certain amount of incompatibility between the selected genotypes. F_1 fruits were slightly elongated than resistant parent, colour of fruit was green with white strips with spine as in resistant parent. This shows that character, fruit length is quantitative in nature and is incompletely dominant. The fruit colour and spiny nature was dominant in this particular parental combination.

Forty F_2 plants (Plate 1d) 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. In BSA plants from 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).

5.3. Phenotyping of genotypes for bacterial wilt incidence

Resistant parent, susceptible parent and F_2 plants of the crossed, resistant parent x susceptible parent were inoculated artificially by stem puncture method and F_1 progenies were screened in sick plots for bacterial wilt incidence and scoring of genotypes as resistant/susceptible was done by Mew and Ho (1976) system. Observations revealed that *Solanum melongena* var. *insanum* I.C. number 421463 was resistant with 85 per cent survival and Pusa Purple Long was susceptible with zero per cent survival. The F_1 plants in sick plot evaluation registered 80 per cent survival. The F_2 plants after artificial inoculation registered 27 per cent survival.

Phenotyping of genotypes with artificial inoculation classified the varieties, 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. The F_1 data suggested that the bacterial wilt resistance gene is incompletely dominant, while F_2 data suggested that it is homozygous recessive. The data generated in hot spot evaluation may not be reliable as it does not ensure entry of pathogen into the system.

The data generated from parental and F_2 generation evaluation was used for genetic analysis of bacterial wilt resistant gene as shown below:

Solanum melongena var. *insanum*
I.C. number 421463 (Resistant) x Pusa Purple Long (Susceptible)
(rr) (RR)

F_1 (Rr)

F_2 (RR, Rr, rr) - 73% wilting (Susceptible-RR, Rr)

- 27% survival (rr)

The ratio of susceptible to resistant in F_2 generation is 2.7:1 so it can be assumed that bacterial wilt resistant character in *Solanum melongena* var. *insanum* I. C. number 421463 is homozygous recessive in nature. According to Gopimony (1983) *Solanum melongena* var. *insanum* is resistant to bacterial wilt and is monogenic and dominant. His observation may be true with respect to the particular parental combination viz., *Solanum melongena* var. *insanum* X Pusa Purple Giant. But need further confirmation.

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. 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. 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.

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 resistance 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 mechanism in tomato variety 'Sakthi' was monogenic and incompletely dominant supports the present study. Girijadevi and Peter (1987) made crosses of two hot pepper lines viz., 'Manjai' and 'Pant C-1' with five sweet peppers. All the F_1's were susceptible or moderately susceptible indicating the recessive nature of inheritance of resistance to bacterial wilt. Momma and Sakata (1993) reported that the bacterial wilt resistance was partially recessive as there was incomplete dominance towards susceptibility.

Markose (1996) studied the inheritance of bacterial wilt resistance in chilli 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 *viz.,* 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 forty F_2 plants were isolated prior to the artificial inoculation. DNA of five susceptible and resistant genotypes for bulking was selected after obtaining the screening data for bacterial wilt resistance.

5.4. Molecular characterization of brinjal genotypes with RAPD marker

Molecular characterization of selected brinjal genotypes and F_2 segregating population for tagging bacterial wilt resistant gene was done with RAPD (Random Amplified Polymorphic DNA) and STS (Sequence-Tagged Site) markers.

5.4.1. Standardization of genomic DNA isolation

Isolation of good quality genomic DNA is one of the most important prerequisites for doing RAPD and STS analysis. The CTAB method with 1x extraction buffer and washing solution of 76 per cent ethanol/containing 10 mM Ammonium acetate yielded good quantity DNA from tender leaves. Few samples showed RNA contamination. RNA was removed by RNase treatment (Nunome *et al*., 2001.) (Plate 3b). The DNA isolated thus appeared as clear, single intact band of high intensity, without shearing, free of RNA and protein contamination in agarose gel. The ratio of UV absorbance ($A_{260}/_{280}$) ranged between 1.80-1.99.

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 repotted that the use of young leaves was best for DNA isolation for RAPD assay.

Micheli *et al*. (1994) reported that RNA in the genomic DNA preparation often influences the reproducibility of RAPD patterns. So, removal of RNA contamination was preferred.

5.4.1.1. Isolation DNA for RAPD analysis

DNA for RAPD analysis was isolated as per standardized procedure from resistance parent, susceptible parent and 40 F_2 progenies. DNA isolation of F_2 progenies was done prior to screening them against bacterial wilt incidence. After obtaining the screening data the DNA of the F_2 progenies were categorized as resistant and susceptible ones. The quality and quantity of isolated DNA samples are presented in Table 6. The ratio of UV absorbance ($A_{260}/_{280}$) ranged between 1.80-1.99. The quantity of DNA in the isolated samples ranged from 3794.49 to 4702.84 ng/µl. Isolated DNA was dispersed in TE buffer for long term use and stored at -20^{0}C for RAPD, bulk segregant and STS analysis.

5.4.2. RAPD (Random Amplified Polymorphic DNA) analysis

The RAPD technique was developed by Williams *et al.* (1991). This simple technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary 10-12 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). Usually RAPD markers are dominant in nature (Waugh *et al.*, 1992) because polymorphisms are detected as the presence or absence of bands. This procedure is rapid, requires only small amounts of DNA, which need not be of high quality.

5.4.2.1. Screening of primers for RAPD analysis

Ninety eight decamer primers in the series of A, AG, OPA, OPAG, OPAH, OPB, OPC, OPF, OPG, OPH, OPL, OPM, OPP, OPU, PUC, R, RA, RF, RN, RY, S, SC and WG as shown in (Table 7 to Table 13) were screened for DNA amplification. Among them, thirty were wilt specific primers. The template DNA was of resistant parent and susceptible parent. The amplified products were run on

agarose gel. The documented profiles were examined for polymorphism and number of amplicons produced. Out of the total primers screened, seventeen has given polymorphism between resistant and susceptible parents. These were selected for BSA. None of the wilt specific primers has given polymorphism between resistant and susceptible parents.

Initial screening of primers with template DNA of resistant and susceptible parent minimized the labour and input required for total RAPD analysis.

5.4.2.2. Bulked Segregant Analysis (BSA)

BSA was done with seventeen selected primers with resistant parent, susceptible parent, resistant bulk (5 resistant F_2 progenies), susceptible bulk (5 susceptible F_2 progenies), negative control without DNA and marker. Resistant bulk and susceptible bulk consisted of F_2 plants originated from a single F_1 by selfing. The primers used for BSA were A-12, OPA-23, OPA-26, OPA-29, OPA-32, OPAH-06, OPC-09, OPP-14, OPU-03, OPU-09, RN-11, RN-12, RN-18, RN-19, RY-01, RY-11 and RY-14 (Table 14). The amplified products were electrophorized and documented (Plate 13-17). Out of the seventeen primers screened for polymorphism, only OPP-14 produced a polymorphic amplicon of size 470 bp in resistant parent and resistant bulk which was absent in susceptible parent and susceptible bulk (Plate 14b).

BSA 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 which linked loci might be detected as polymorphic between the bulked samples. Generally the sample size for BSA ranges from 5-15 number (Wang *et al.* 1995).

Sixteen primers which had given polymorphism in primer screening did not give same results in BSA. 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 10-mer 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. 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. These report suggest that RAPD results are sensitive to PCR condition like annealing temperature, GC content, single nucleotide change, concentration of polymerase and quality and quantity of DNA (Williams *et al.*, 1990; Fakuoka *et al.*, 1992; Murray and Thompson, 1994).

5.4.2.3. Co-segregation analysis

Co-segregation analysis was carried out with resistant parent, susceptible parent; individuals of resistant and susceptible bulk F_2's with selected primer OPP-14. The result is presented in Plate 18. The band of 470 bp was present in resistant parent, 5 resistant F_2's and three susceptible F_2's. It was absent in susceptible parent and two susceptible F_2's.

In co-segregation analysis OPP-14 amplicon of 470 bp did not segregate as expected. Identified marker may not be able to distinguish resistant homozygous recessive and susceptible heterozygote plants. It is told that RAPD is a dominant marker. The marker RAPD was selected for tagging bacterial wilt resistance gene in resistant parent *Solanum melongena* var. *insanum* I. C. number 421463 based on the report of Gopimony (1983). According to him the controlling gene is monogenic and dominant. In the present study resistant parent included was not the same specific accession, which he had used.

BSA and co-segregation analysis reduces the error associated with molecular markers in distinguishing polymorphism among resistant and

usceptible individuals. Co-segregation analysis cross checked the information produced by bulk segregant analysis.

5.5. Molecular Cloning

The specific polymorphic amplicon of 470 bp in resistant genotypes was eluted from the resistant parent specific lane (Plate 19a) using gel elution kit (section number 3.2.3.7.2.). The bacterial *E. coli* JM109 strain was used for cloning of eluted product. The competency JM 109 for transformation was checked by introducing plasmid pUC18 containing selectable marker ampicillin and *LacZ* are reporter gene. Transformed cells appeared as blue colonies (Plate 20). The eluted product was ligated with pGEMT vector containing ampicilin resistance gene and *LacZ* gene as markers. pGEMT vector system containing the eluted product was used for transforming the competence cells of JM 109. Multiple copies of eluted product was made in transformed *E. coli* JM 109 strain (Plate 22).

The transformed colonies appeared as white due to insertional inactivation of *LacZ* gene sequences. The cloning of eluted amplicon was necessary for getting sufficient copies for sequencing.

5.6. Confirmation of DNA insert by colony PCR

Confirmation of insert was carried out by colony PCR amplification of recombinant and non-recombinant plasmid DNA using T7 forward and SP6 reverse universal primers as described in section 3.2.3.10. Amplicons for blue and white colonies differed in their size. The PCR product of blue colony plasmid had lower molecular weight with 175 bp since it lacked the insert and only T7 and SP6 regions got amplified. The PCR product of white colonies gave higher molecular weight band of 700 bp as the insert also got amplified (Plate 24) along with T7 and SP6 regions of the vector. The positive controls for white colonies produced 750 and 175 bp bands respectively.

5.7. Sequencing of the cloned fragment

The prepared white colony stab, *W-3* was sequenced by automated sequencing with T7 primers and provided 5'-3' sequence data in forward directions with precise order of nucleotides. Sequence information obtained from *W-3* clone was referred as *W-3*. The sequence data of *W-3*, 700 bp was further analyzed by various bioinformatics tools for getting more sequence information.

5.8. *In silico* analysis of sequences

5.8.1. Vector screening

The sequence obtained after automated sequencing subjected to vector screening using the VecScreen tool in NCBI to remove vector region from clone.

A sequence of 408 bp was obtained from *W-3* after VecScreen and was named *"Sol-3*" (Fig 5.). This was used for STS primer designing and analysis of other bioinformatics tools.

5.8.2. Nucleic acid and Protein sequence analysis

4. **Nucleotide- nucleotide BLAST (Blastn)**

The nucleotide sequence of "*Sol-3*" was compared with the sequences available in nucleotide database using Blastn tool (www.ncbi.nlm. nih.gov/blast/; Altschul *et al*., 1997) provided by NCBI. The result is shown in shown in Fig 6. Blastn search showed homology with AC237888 *Solanum tuberosum* clone RH183L22 published sequences present in NCBI database. It has shown 77 per cent identity to AC237888 *Solanum tuberosum* clone RH183L22.

2. Protein- protein BLAST (Blastp)

The nucleotide sequence of "*Sol-3*" was translated into amino acids using EMBOSS Transeq tool (http://www.ebi.ac.uk/Tools/emboss/transeq/). The result obtained is given as Fig 7. After removing the non-coding sequences, the amino

acids sequences were subjected to homology search for known protein with Blastp. It has showed homology with ABC948993 polyprotein (*Oryza australiesis*) S=76 E=1.6e-14 present in NCBI database with 76.3 total score.

3. Detection of Open Reading Frame (ORF)

Presence of ORF in "*Sol-3*" was searched with bioinformatics tool ORF finder. It revealed four ORFs, located on the plus strands (+1, +1 and +3) and one was located on minus (-2) (Fig.9). The ORF on +3 frame with a longest length of 108bp revealed significant level of homology with putative retrotransposon protein in *Solanum demissum* with 88% query coverage and 71% of identity with published sequences present in NCBI database.

5.9. Development of STS marker

5.9.1. STS primer designing

Two Sets of 22 bp length STS forward and reverse primers were designed from the sequence information of cloned OPP-14 RAPD fragment, "*Sol-3*" using Primer3Plus tool. They were named as BWRGB-1 and BWRGB-2. The criteria considered in designing the primer are described in Section 3.2.4.1. The details of primers are given Table 15 and Fig.10.

5.9.2. PCR analysis of STS primers

The DNA from resistant parent (*Solanum melongena* var. *insanum* I.C. number 421463), susceptible parent (Pusa Purple Long), resistant bulk, and susceptible bulk were amplified with STS primer Set-2 BWRGB-2. A negative control was also kept without template DNA. PCR was done as detailed in (section 3.2.4.3.) Mastercycler and Gradient Mastercycler. The amplified products were electrophorised on 2 per cent agarose gel (Plate 25). STS primers amplified 200 bp in all samples tested. The designed STS primers were not able to distinguish resistant and susceptible genotypes.

In co-segregation analysis OPP-14 has amplified bacterial wilt specific 470 bp amplicon in resistant parent, resistant bulk and in two susceptible F_2's . So the STS primer designed from OPP-14 may not be possible to distinguish related homozygous recessive and heterozygous susceptible ones.

Future lines of work

- Design more STS primers and amplify resistant and susceptible genotypes to explore the possibility of getting polymorphism.
- Thorough screening of parent, F_1 and F_2 progenies with more population to know more about genetic control of bacterial wilt resistance in *Solanum melongena* var. *insanum*.
- Molecular characterization can be done with co-dominant markers like SSR and RFLP which could distinguish homozygous dominant from heterozygous dominant.

Summary

6. SUMMARY

The study on 'Tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* by molecular markers' was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period from 2009 to 2011. The main objective of the study was to identify molecular marker linked to bacteria wilt resistance in *Solanum melongena* var. *insanum*. The genotypes used for study were resistant variety *Solanum melongena* var. *insanum* I. C. number 421463, susceptible variety Pusa Purple Long, F_1 and F_2 progenies of cross *Solanum melongena* var. *insanum* I. C. number 421463 x Pusa Purple Long.

The salient findings of the study are summarized below:

i. F_1 plant was raised by crossing resistant variety *Solanum melongena* var. *insanum* I.C. number 421463 as the female parent and susceptible variety Pusa Purple Long as the male parent. The fruit setting in parental cross 4 per cent and selfing of F_1 plants was 11 per cent. Segregating F_2 progenies for bacterial wilt incidence was raised by selfing single F_1 plant.

ii. The parents and F_2 progenies were screened for bacterial wilt incidence by artificial inoculation of *Ralstonia solanacearum* inoculum through stem puncture method. The severity of wilt incidence was scored according to scoring system by Mew and Ho (1976). *Solanum melongena* var. *insanum* I. C. number 421463 was resistant with 85 per cent survival. The Pusa Purple Long was scored as susceptible (S) as survival percentage was zero. The F_2 population was categorized as susceptible (S) with 27 per cent survival. In F_2 generation, ratio of susceptible to resistant was 2.7:1 and was assumed that bacterial wilt resistant character in var. *insanum* is homozygous and recessive.

iii. The CTAB method with 1x extraction buffer and washing solution of 76 per cent ethanol/containing 10 mM ammonium acetate yielded good quantity DNA from tender leaves. RNA contamination was removed by RNase treatment (Nunome *et al*., 2001.). The ratio of UV absorbance ($A_{260}/_{280}$) ranged between 1.80-1.99. The quantity of DNA in the isolated samples ranged from 3163.93 to 4668.11 ng/µl.

iv. Primer screening was done with ninety eight 10-12 bp primers were screened with resistant and susceptible parents. Among them Thirty were wilt specific primers reported in others crops. Out of ninety eight primers, seventeen were selected for BSA based on polymorphism between resistant and susceptible parents. None of the wilt specific primers showed polymorphism.

v. BSA was done with the DNA of resistant parent, susceptible parent, resistant and susceptible bulk. Out of the seventeen primers screened for polymorphism, only OPP-14 produced a polymorphic amplicon of size 470 bp in resistant parent and resistant bulk which was absent in susceptible parent and susceptible bulk.

vi. Co-segregation analysis of individuals of resistant bulk and susceptible bulk with OPP-14 primer revealed its presence in resistant parent, resistant F_2 and three susceptible F_2 progenies.

vii. The polymorphic 470 bp band amplified by OPP-14 was eluted from the resistant parent and ligated with pGEMT vector. The ligated product was cloned in competent cells of *E. coli* JM 109. The white colonies in LBA medium containing X-gal and IPTG represent the transformed ones.

viii. Confirmation of insert was carried out by colony PCR using T7 forward and SP6 reverse universal primers. The higher molecular weight amplicons of 700 bp from white colonies against 175 bp band in blue colonies confirmed presence of insert in white ones.

ix. Stab was made from transformed white colonies and was sequenced in automatic sequencer with T7 universal primer. The sequenced *W-3* had 700 bp length.

x. Vector screening was performed to remove the vector sequence from *W-3* using the VecScreen tool in NCBI and yielded 408 bp. This was named as *"Sol-3"*.

xi. *"Sol-3"* sequence was subjected to Blastn search. It revealed significant level of homology with AC237888 *Solanum tuberosum* clone RH183L22 published sequences present in NCBI database with 77 per cent identity

xii. *"Sol-3"* nucleotide sequence was subjected to EMBOSS Transeq tool and Blastp search. It revealed significant level of homology with ABC948993 polyprotein (*Oryza australiesis)* S=76 E=1.6e-14 present in NCBI database with 76.3 total score.

xiii. *"Sol-3"*sequence was subjected to ORF finder and revealed four ORF frame. The longest ORF, 108 bp on +3 frame revealed significant level of homology with putative retrotransposon protein in *Solanum demissum* with 88 per cent query coverage and 71 per cent of identity with published sequences present in NCBI database.

xiv. Two Sets of 22 bp STS primers forward and reverse (BWRGB-1 and BWRGB-2) with a melting temperature of 57.7 ^{0}C and 57.4 ^{0}C and GC content of 40-60 per cent was designed from the sequence information of cloned RAPD OPP-14 amplicon, *"Sol-3"*using Primer3Plus tool.

xv. PCR amplification of DNA from resistant parent, susceptible parent, resistant bulk, and susceptible bulk with STS primer BWRGB-2 was done with specific PCR conditions. STS primer amplified approximately 200 bp in all the genotypes tested. It was not able to distinguish resistant and susceptible genotypes.

ABSTRACT

The study entitled 'Tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* by molecular markers' 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 1896, Yabuuchi *et al.*, 1995) is an important problem in cultivation of tropical and subtropical crops like potato, brinjal, chilli, tomato etc. World wide approach to control the disease is to use resistant varieties. Gopimony (1983) reported *Solanum melongena* var. *insanum* (wild variety) is resistant to bacterial wilt and controlling gene is monogenic and dominant.

This investigation was taken up for tagging of bacterial wilt resistance gene in *Solanum melongena* var. *insanum* by RAPD through bulked segregant analysis as reported by Michelmore *et al.*, (1991). The genotypes used for the study were resistant variety *Solanum melongena* var. *insanum* I.C. number 421463, susceptible variety Pusa Purple Long and segregating F_2 population for bacterial wilt incidence. To raise segregating F_2 population F_1 was raised by controlled crossing of *Solanum melongena* var. *insanum* I.C. number 421463 with pollen grains of Pusa Purple Long. Then F_1 plant was selfed to get F_2 population.

The parents and F_2 genotypes were screened for bacterial wilt incidence by stem puncture method of artificial inoculation using *R. solanacearum* inoculum. In all cases death of plants were confirmed by ooze test. The genotypes were classified against bacterial wilt incidence according to classification of Mew and Ho (1976). The variety *Solanum melongena* var. *insanum* I. C. number 421463 was resistant, Pusa Purple Long and F_2 were susceptible. Ratio of susceptible to resistant in F_2 generation was 2.7:1. So it can assume that bacterial wilt resistant character in resistant parent can be homozygous and recessive.

Genomic DNA for RAPD analysis was isolated by modified CTAB 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 standardized. Ninty eight primers belonging to series A, OPA, OPAG, OPAH, OPB, OPC, OPF, OPP, OPU, RY and RN were initially screened with DNA of resistant and susceptible parents to select primers with polymorphism. Out of ninty eight primers tested thirty, were reported as bacterial wilt specific. Seventeen primers were selected for BSA based on polymorphism. None of wilt specific primers showed polymorphism.

Bulked segregate analysis was done with seventeen selected primers for polymorphism. The genotypes used for the study were susceptible parent, resistant parent, five resistant and five susceptible F_2 progenies. Among the tested primers only OPP-14 has recorded polymorphism between resistant and susceptible genotypes in BSA. It has produced 470 bp polymorphic amplicon in resistant parent and resistant bulk. Co-segregation analysis was done with OPP-14 primer with individuals of susceptible and resistant bulk. In co-segregation analysis OPP-14 specific amplicon got amplified not only in resistant parent, resistant bulked but also in three susceptible F_2s also.

The polymorphic band produced by OPP-14 primer in resistant parent and resistant bulk was eluted from resistant parent and cloned in pGEM-T vector, and was transformed into *E. coli* JM 109 cells. Recombination of the insert was confirmed through colony PCR reaction with universal T7 and SP6 primers. The cloned fragment was sequenced to obtain the nucleotide sequence information and was named as *W-3*.

The sequence obtained after vector screening was named as "*Sol-3",* was subjected to Blastn search. Blastn search revealed significant levels of homology with AC237888 *Solanum tuberosum* clone RH183L22 present in NCBI database. The sequence was also analysed using EMBOSS Transeq and Blastp. Analysis revealed homology with ABC948993 polyprotein (*Oryza australiesis*). ORF

analysis revealed the longest ORF of 108 bp was on +3 frame which revealed significant level of homology with putative retrotransposon protein in *Solanum demissum*.

Two sets STS primers were designed using Primer3Plus. The efficiency of STS primer set BWRGB-2 to distinguish resistant and susceptible genotypes was tested and it amplified a fragment of 200 bp in all the genotypes tested.

References

RERENCENCES

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

Ajjappalavara, P. S., Dharmatti, P. R., Salimath, P. M., Patil, R. V., Patil, M. S., and Krishnaraj, P. U. 2008. Genetics of bacterial wilt resistance in brinjal karnataka. *J. Agric. Sci.* 21: 424-427.

Akiba, F., Riberio, R. D., Sudo, S., Robbes, C. F., and Kimura, D. 1972. The evaluation of introduced tomato strains for susceptibility to Brazilian isolates of *Pseudomonas solanacearum*, the agent of bacterial wilt. *Arguivos do Instituto. Biol*. 39: 243-247.

Altschul, S. F., Thomas, L. M., Schaffer, A. A., Zhang, J., Zhang, S., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402.

Andres, A. M., Ortega, G. R., Arteaga, L. M., and Hormaza, J. 2004. Development of RAPD and SCAR markers linked to the *Pvr4* locus for resistance to PVY in pepper (*Capsicum annuum* L.). *Theor. Appl. Genet*. 105(6): 1067-1074.

Archak, S., Karihaloo, J. L., and Jain, D. 2002. RAPD markers reveal narrowing of genetic base of Indian tomato cultivars. *Curr. Sci.* 82 (9): 1139-1145.

Arlat, M., Gough, C. L., Zischek, C., Barberis, P. A., Trigalet, A., and Boucher, C. 1992. Transcriptional organization and expression of the large hrp gene cluster in *Pseudomonas solanacearum*. *Mol. Microbiol.* 6:3065–3076.

AVRDC. 1975. Genetic study of resistance to bacterial wilt. *Tomato Reptr*. 24-25.

Ayres, N. M., McClung, A. M., Xarkin, P. D., Bligh, H. F. J., Jones, C. A., and Park, W. D. 1997. Microsatellites and a single nucleotide polymorphism differentiate apparent amylase classes in an extended pedigree of US rice germplasm. *Theor. Appl. Genet*. 94: 773-781.

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

Balatero, C. H., Hautea, D. M., and Narciso, J. O. 2000. Genetic of resistance and host pathogen interaction in tomato *P. solanacearum* system: implications in breeding for tomato. *Philipp. J. Crop. Sci.* 25:8.

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

Benjamin, B. and Burr, F. A. 1999. Recombinant inbred for molecular mapping in maize: Theoretical and practical considerations. *Trends Genet*. 7: 55-58.

Bi-hao, C., Jian-jun, L., Yong, W., and Guo-ju, C. 2009. Inheritance and identification of SCAR marker linked to bacterial wilt-resistance in eggplant. *Afr. J. Biotech*. 8(20): 5201-5207.

Blair, M. W. and McCouch, S. R. 1997. Microsatellite and sequence-tagged site markers diagnostic for the rice bacterial blight resistance gene *xa 5*. *Theor. Appl. Genet*. 95: 174-184

Bosch, S. E., Louw, A. J., and AUCAMP, E. 1985." Rodale" bacterial wilt resitance tomato. *Hort. Sci.* 20:458-459.

Bowles, D. J. 1990. Defense-related proteins in higher plants. *Annu. Rev. Biochem*. 59:873-907.

Buddenhagen, I. W. 1986. Bacterial wilt revisited, *ACIAR Proceedings*, 13, in: G.J. Persley, Editor, *Bacterial Wilt Disease in Asia and the South Pacific*. 126–143.

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

Butranu, W., Yinasawapun, S., and Srithongchai, W. 1994. Status of groundnut bacterial wilt research in Thailand. *Groundnut Bacterial Wilt in Asia*. ICRISAT. Hyderabad. 3:129-133.

Caranta, C., Thabuis, A., and Palloix, A. 1997. Development of a CAPS marker for the *Pvr4* locus: a tool for pyramiding potyvirus resistance genes in pepper. *Genome*. 2(6):1111-1116.

Carmeille, A., Caranta, C., Dintinger, J., Prior, P., Luisetti, J., Besse, P. 2006. Identification of QTLs for *Ralstonia solanacearum* race 3-phylotype II resistance in tomato. *Theor. Appl. Genet*. 113(1):110-121.

Chadha, M. L. 1993. Improvement of brinjal. In: Chadha KL and Kalloo G (eds.), Adv. Horticult. 5. Vegetable crops, part 1, Malhotra, New Delhi, India.105-135.

Chague, V., Mercier, J.C., Guenard, M., de, C. A., and Vedel, F. 1997. Identification of RAPD markers linked to a locus involved in

quantitative resistance to TYLCV in tomato by bulked segregant analysis. *Theor. Appl. Genet*. 95: 671-77.

Chaudhary, D. R. 2000. Inheritanc of resistance to bacterial wilt (*Ralstonia solanacearum* Smith EF) in eggplant. *Haryana J. Hort. Sci.* 29: 89-90.

Chaudhary, D. R. and Sharma, S. D. 2000. Screening of some brinjal cultivars against bacterial wilt and fruit borer. *Agril. Sci. Dig*. 20(2):29-30.

Chawla, H. S. 2002. *Introduction to Plant Biotechnology*. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi. 538p

Che, N. C., Li, H. M., and Wang, J. F. 1997. Bacterial wilt resistance sources in eggplant, *Solanum melongena*. *Capscicum* and Eggplant News, 16: 111-114.

Chester, K. 1933. The problem of acquired physiological immunity in plants. *Quart. Rev. Biol.* 8:129-151.

Chunwongse, J., Bunn, T. B., Crossman, C., Jians, C., and Tansley, J. 1994. Chromosomal localization and molecular marker tagging of powdery mildew resistant gene (Lv) in tomato. *Pl Sci.* 89 (1): 76-79.

Chunwongse, J., Doganlar, S., Crossman, C., Jiang, J., and Tanksley, S. D. 1997. High-resolution genetic map the Lv resistance locus in tomato. *Theor. Appl. Genet*. 95:220–223.

Clain, D., Silva, D., Fock, S., Vaniet, A., Carmeille, C., Gousset, D., Sihachakr, J., Luisetti, H., Kodja, L., and Besse, P. 2004. RAPD genetic homogeneity and high levels of bacterial wilt tolerance in

Solanum torvum Sw. (Solanaceae) accessions from Reunion Island. *Pl. Sci.* 166 (6): 1533-1540.

Collins, N. C., Lahaye, S., Peterhänsel, C., Freialdenhoven, A., Corbitt, M., and Schulze-Lefert, P. 2001. Sequence Haplotypes Revealed by Sequence-Tagged Site Fine Mapping of the *Ror1* Gene in the Centromeric Region of Barley Chromosome 1H. *Pl. Physiol.* 125(3): 1236–1247.

Collonnier, C., Fock, I., Kashyap, V., Rotino, G. L., Daunay, M. C., Lian, Y. Mariska, I. K. Rajam, M.V., Servaes, A., Ducreux, G. and Sihachakr, D. 2001. Applications of biotechnology in eggplant. *Pl.Cell, Tissue Organ Cul.* 65: 91–107.

Danesh, D. and Young, N. D. 1994. Partial resistance loci for tomato bacterial wilt show differential race specificity. *Tomato Genet. Crop Rep.* 44:12-13

Darby, G. and Brown, C. A. 2004. *Ralstonia solanacearum* genes induced during growth in tomato: an inside view of bacterial wilt. *Mol. Microbiol.* 53(6):1641–1660.

Darvasi, A. and Soller, M. 1994. Optimum pacing of genetic markers for determining linkage between marker loci and Quantitative Trait Loci. *Theor. Appl. Genet.* 89: 351-353.

Demir, K., Bakir, M., Sarikamu, G., and Acunalp, S. 2010. Genetic diversity of eggplant (*Solanum melongena*) germplasm from Turkey assessed by SSR and RAPD markers. *Genet. Mol. Res.* 9 (3): 1568-1576.

Deslandes, L., Olivier, J., Theulieres, F., Hirsch, J., Feng, D. X., Bittner-Eddy, P., Beynon, J., and Marco, Y. 2002. Resistance to Ralstonia

solanacearum in Arabidopsis thaliana is conferred by the recessive *RRS1-R* gene, a member of a novel family of resistance genes. *Proc. Natl. Acad. Sci.* 99:2404-2409.

Divakaran, A. 2007. Molecular characterization of tomato (*Solanum lycopersicon* L.) with special reference to tomato leaf curl virus (ToLCV) resistance. M.Sc. Ag) thesis, Kerala Agricultural University, Thrissur. 87p.

Doganlar, S., Frary, A., Daunay, M. C., Lester, R. N., and Tanksley, S. D. 2002. A comparative genetic linkage map of eggplant (*Solanum melongena*) and its implications for genome evolution in the Solanaceae. *Genet*. 161:1697–1711

Ellis, J., Dodds, P., and Pryor, T. 2000. Structure, function and evolution of plant disease resistance genes. *Curr. Opin. Plant Biol*. 3: 278–284.

Fegan, M. and Prior, P. 2005. How complex is the Ralstonia solanacearum species complex. In Bacterial Wilt Disease and the Ralstonia solanacearum Species Complex ed. Allen, C., Prior, P. and Hayward, A.C. pp. 449–462. Madison: APS Press.

Feng, L. L., Qu, D.Y., Jin, L. P., and Lian, Y. 2003. Genetic analysis of resistance to bacterial wilt (*Ralastonia solanacearum*) in eggplant (*Solanum melongena* L.). *Acta Hort. Sinica*. 30(2): 163-166.

Ferrer Z., A. 1974. Resistance to *Pseudomonas solanacearum* in *Lycopersicon esculentum*. PhD thesis. Univ. of Florida.

Flavier, A. B, Schell, M. A., and Denny, T. P. 1998. An *RpoS* (rs) homologue regulates acylhomoserine lactone-dependent autoinduction in *Ralstonia solanacearum*. *Mol. Microbiol.* 28(3):475–486.

Fock, I., Collonnier, C., Purwito, A., Luisetti, J., Souvannavong, V., Vedel, F., Servaes, A., Ambroise, A., Kodja, H., Ducreux, G., and Sihachakr, D. 2000. Resistance to bacterial wilt in somatic hybrids between *Solanum tuberosum* and *Solanum phureja*. *Pl. Sci.* 160:165-176.

French, E. R. and De Lindo, L. 1982. Resistance to *Pseudomonas solanacearum* in potato: specificity and temperature sensitivity. *Phytopathology*. 72 pp. 1408–1412

French, E. R., Anguiz, R., and Aley, F. P. 1998. The usefulness of potato resistance to *Ralstoniasolanacearum*, for the integrated control of bacterial wilt, P. Prior, C. Allen, J. Elphinstone, Editors , *Bacterial Wilt Disease, Molecular and Ecological Aspects*, Springer, Berlin (1998), pp. 381–385.

Fu, Y., Rowland, B. G. G., Duguid, S. D. and K. W. Richard. 2003. RAPD analysis of 54 North American flax cultivars. *Crop Sci.* 43: 1510-1515.

Fukuoka, S., Hosaka, K. and Kamijima, O. 1992. Use of random amplified polymorphic DNAs (RAPDs) for identification of rice accessions. *Jpn. J. Genet*. 67: 243-252.

Furini, A. and Wunder, J. 2004. Analysis of eggplant (*Solanum melongena*) - related germplasm: morphological and AFLP data contribute to phylogenetic interpretations and germplasm utilization. *Theor. Appl. Genet*. 108: 197–208.

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

Gangashetti, M. G., Jena, K. K., Shenoj, V. V., and Freeman, W. H. 2004. Inheritance of elongated uppermost internode and identification of RAPD marker linked to *eui* gene in rice. *Curr. Sci.* 87 (4): 469-475.

Geetha, P.T. and Peter, K. V. 1993. Bacterial wilt resistance in a few selected lines and hybrids of brinjal (*Solanum melongena* L.). *J. Trop. Agric*. 31(2): 274-276.

Ghosh, P. P. and Mandal, N. C. 2009. Some disease management practices for bacterial wilt of potato. *J. Pl. Prot. Sci.* 1(1): 51-54.

Gilbert, J. C., Tanaka, J. S., and Takeda, K. Y. 1974. Kewalo tomato. *Hortic. Sci.* 9: 481-482.

Girijadevi, T. and Peter, K. V. 1987. Field resistance of capsicum hybrid Yolo Wonder Improved x KAU Cluster to bacterial wilt. *Agric. Res. J. Kerala*. 25: 293-294.

Gokulapalan, C., Mini, C., Krishnakumar, K., Sureshbabu, K.V., Mathew, S. K., and Sadhankumar, P. G. 2004. Solanaceous vegetables. In: Gopalakrishnan, T.R (ed).*Three Decades of Vegetable Research in Kerala Agricultural University*. Kerala Agricultural University. Thrissur. pp 5-17.

Gonzalez, W. G., and Summers, W. L. 1995. A comparison of Pseudomonas solanacearum resistant tomato cultivars as hybrid parents. *J. Am. Soc. Hort. Sci.* 120:891–895

Gopalakrishnan, T. R., Singh, P. K., Sheela, K. B., Shankar, M. A., Kutty, P. C. J., and Peter, K. V. 2005. Development of bacterial wilt resistant varieties and basis of resistance in eggplant (*Solanum melongena* L.), In: Allen C, Prior P & Hayward A (eds.) Bacterial

wilt disease and the *Ralstonia solanacearum* species complex, APS Press, St Paul, pp. 293-300.

Gopimony 1983. Genetic studies in brinjal relation to bacterial wilt resistance. Ph.D. (Ag) Thesis. Kerala Agricultural University, Thrissur. 195p

Gopinath, G., Madalageri, B. B. 1986. Bacterial wilt (*Pseudomonas solanacearum* Smith EF) resistance in eggplant. *Veg. Sci.* 13: 189-195.

Gousset, C., Collonnie, C., Mulya, K., Mariska, I., Rotino, G. L., Besse, P., Servaes, A., and Sihachakr, D. 2005. *Solanum torvum*, as a useful source of resistance against bacterial and fungal diseases for improvement of eggplant (*Solanum melongena* L.). *Pl. Sci.* 168: 319-327.

Gousset, C., Lian, Y., Mariska, I., Rotino, G., Servaes, A., and Sihachakr, D. 2004. Determination of the inheritance of resistance to bacterial wilt(*Ralstonia solanacearum*) in eggplant (*Solanum melongena*). Proceedings of the 12th Eucarpia Meeting on Genetics and Breeding of *Capsicum* and Eggplant, p. 183.

Gowha, P. H. R., and Shivasbankara, K. T. 1990. Intervarietal hybridization in brinjal for bacterial wilt resistance. *Current Res. United Agric. Sci.* 19(4):70–71.

Graham, K. M. and Yap, T. C. 1976. Studies on bacterial wilt inheritance of resistance to Pseudomonas solanacearum in tomato. *Malays Agric. Res* 5(1):1–8

Grilli, G. V. G., Braz, L. T., and Lemos, E. G. M. 2007. QTL identification for tolerance to fruit set in tomato by fAFLP markers. *Crop Breed. Appl. Biotech.* 7: 234-241.

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

Grimley, N. and Hanson, P. 1998. Genetics of plant resistance to bacterial wilt: round table report, P. Prior, C. Allen, J. Elphinstone, Editors , *Bacterial Wilt Disease, Molecular and Ecological Aspects*, Springer, Berlin, pp. 263–266.

Gupta, P.K and Varshney, R. K. 2000. The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. *Euphytica*. 113:163-185

Hanson, P. M., Licardo, O., Hanudin, M., Wang, J. F., and Chen, J. T. 1998. Diallel analysis of bacterial wilt resistance in tomato derived from different sources. *Pl. Dis.* 82(1): 74-78.

Hanson, P. M., Wang, J. F., Licardo, O., Hanudin, Mah, S. Y., Hartman, G.L., Lin, Y. C. and Chen, J. T. 1996. Variable reaction of tomato lines to bacterial wilt evaluated at several location in Southeast Asia. *Hort. Sci.* 31:143-146.

Hawkes, J. G. The Potato. Evolution, Biodiversity and Genetic Resource, Belhaven Press, London, 1990, 259 pp.

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

Hayward, A. C., El-Nashaar, H. M., Nydegger, U., and De Lindo, L. 1990. Variation in nitrate metabolism in biovars of *Pseudomonas solanacearum*. *J. Appl. Bact*. 69:269–280.

Hayward, A.C. 2000. *Ralstonia solanacearum*. In Encyclopedia of Microbiology, Vol. 4 ed. Lederberg, J. pp. 32–42. San Diego: Academic Press.

He, H. Y., Sequeira, L., and Kelman,A. 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. *Pl. Dis*. 67:1357-1361.

Hernandez, P., Martin, A., and Dorado, G. 1999. Development of SCAR's by direct sequencing of RAPD products: a practical tool for the introgression and marker-assisted selection of wheat. *Mol. Breed*. 5: 245-253.

Hikichi, Y., Nakazawa-Nasu, Y., Kitanosono, S., Suzuki, K., Okuno, T. 1999. The behavior of Lux-marked *Ralstonia solanacearum* in grafted tomato cultivars resistant or susceptible to bacterial wilt. *Ann. Phytopathology. Soc. Jpn*. 65:597–603.

Hittalmani, S., Mew, T., Foolad, M. R., and Huang, N. 1995. Identification of blast resistance gene *Pi*-2(t) in a segregating population of Rice. *Theor. Appl. Genet*. 91:9–14.

Hoffman, D., Hang, A., Larson, S., and Jones, B. 2003. Conversion of an RAPD marker to an STS marker for barley variety identification. *Pl. Mol. Biol. Rep*. 21:81-91.

Hurbert, J.J. and Helton, A.W. 1967. A translocated resistance phenomenon in *Prunus domestica* induced by initial infection with *Cytospora cineta*. *Phytopathology*. 57:1094-1098.

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

Hussain, M. Z., Rahman, M. A., and Bashar, M. A. 2005. Screening of brinjal accessions for bacterial wilt caused by *Ralstonia solanacearum*. *Bangladesh. J. Bot*. 34(1):53-58.

James, D. 2001. Molecular characterization of *Ralstonia solanacearum* (Smith) Yabuuchi et al causing bacterial wilt in solanaceous vegetables. M.Sc. (Ag) Thesis, Kerala Agricultural University, Thrissur. 104p.

Janse, J. D., Van den Beld H. E., Ephinstone, J., Simpkins, S., Tjou-Tam Sin, N. N. A. and van Vaerenbergh, J. 2004. Introduction to Europe of Ralstonia solanacearum biovar 2, race 3 in Pelargonium zonale cuttings. *J. Pl. Path*. 87:147–155.

Jaunet T. X., Wang J. F. 1999. Variation in genotype and aggressiveness of Ralstonia solanacearum race 1 isolated from tomato in Taiwan. *Phytopathology*. 89:320-327.

Jena, K. K., Khush, G. S., and Kochert, G. 1992. RFLP analysis of rice (*Oryza sativa* L.) introgression lines. *Theor. Appl. Genet*. 84: 608-616.

Jeon, Y. H., Ahn, S. N., Choi, H. C., Hahn, T. R., and Moon, H. P. 1999. Identification of a RAPD marker linked to a brown plant hopper resistance gene in rice. *Euphytica*. 107(1): 23-28.

Jessykutty, P. C. 1985. Realized selection responses under four methods of selection in 3rd And 4th cycles in a set of brinjal lines. MSc (Ag) thesis, Kerala Agricultural University, Thrissur. 104p.

Johnson, W. C., Guzman, P., Mandala, D., Mkandawire, A. B. C., Temple, S., Gilbertson, R. L., and Gepts, P. 1997. Molecular tagging of the *bc-3* gene for introgression into Andean common bean. *Crop Sci*. 37: 248-254.

Julio, E., Verrier, J. L. and Borne, F. D. D. 2006. Development of SCAR markers linked to three disease resistances based on AFLP within *Nicotiana tabacum* L. *Theor. Appl. Genet*. 112: 335-346

Kang, Y. H. J., Mao, M., He, L., and Schell, M. A. 1994. Dramatically reduced virulence of mutants of *Pseudomonas solanacearum* defective in export of extracellular proteins across the outer membrane. *Mol. Pl. Microbe Interact* 7:370–377

Karumannil, S. 2007. Development of trait related marker for bacterial wilt resistance in tomato (*Solanum lycopersicon* L.). M.Sc. (Ag) thesis. Kerala agricultural University, Thrissur. 115p.

Kasai, K., Morikawa, Y., Sorri, V.A., Valkonen, J. P. T., Gebhardt, C., and Watanabe, K. N. 2000. Development of SCAR markers to the PVY resistance gene *Ryadg* based on a common feature of plant disease resistance genes. *Genome*. 43:1-8

KAU. 2007. *Package of Practices Recommendations Crops 2002*. Directorate of Extension, Kerala Agricultural University, Thrissur. 278p

Kelman, A. 1953. The bacterial wilt caused by *Pseudomonas solanacearum*.*North Carolina Agric. Exp. Stn. Tech. Bull.* 99: 194p.

Kim, H. J., Han, J. H., Yoo, J. H., Cho, H. J. and Kim, B. D. 2008. Development of a sequence characteristic Amplified Region marker

linked to the *L4* Locus conferring Broad Spectrum Resistance to Tobamo viruses in pepper plants. *Mol. Cells.* 25(2): 1-1

Kishun, R. and Chand, R. 1988. Effect of bleaching powder on *Pseudomonas solanacearum* and other soil microflora. *J. Soil Biol. Ecol.* 8: 90-94

Kochert, G., Halward, T., Branch, W. D., and Simpson, C. 1991. RFLP variability in peanut (*Arachis hypogea* L.). *Pl. Mol. Biol*. 18:315-322.

Krausz, J. P. and Thurston, H.D. 1975. Breakdown of resistance to *Pseudomonas solanacearum* in tomato. *Phytopathology*. 65:1272–1274.

Li, H. P., Goth, R. W., and Barksdale, T. H. 1989. Evaluation of resistance to bacterial wilt in eggplant. *Pl. Dis*. 72(5): 437-439.

Li, H. T., Zou, Q. D., Lu, S. W., Mu, X., and Xu, W. K. 2001. The study on bacterial wilt resistance in tomato. *Liaoning Agric. Sci*. 5: 1-4.

Li, H. T., Zou, Q. D., Lu, S. W., Mu, X., Xu, W. K., and Xu, K. H. 2002. Resistance heredity of eggplant to *Pseudomona solanaceanum* Smith EF IIgenetic analysis of eggplant resistance material LS1934. *Liaoning Agric. Sci.* 3: 1-3.

Li, J.T., Yang, J., Chen, D. C., Zhang, X. L. and Z. S. Tang. 2007. An optimized mini-preparation method to obtain high-quality genomic DNA from mature leaves of sunflower. *Genet. Mol. Res.* 6 (4): 1064-1071.

Li, M., Wang, Y. Q., Tian, S. B., Luo, Z. Y., Wang, X. J. 2006. Genetic analysis of resistance to bacterial wilt and identification of an

associated AFLP marker in eggplant (*Solanum melongena*). *Acta Hotic. Sinica*. 33: 869-872.

Lin, C. Y., Hsu, S. T. and Ho, M. C. 1974. Breeding tomato for resistance *to Pseudomonas solanacearum* 1.Inter varietal differentiation in resistance to *Pseudomonas solanacearum*. *Bul. Agric. Res.* 3: 1.

Mackill, D. J. 1996. Classifying Japonica rice cultivars with RAPD marker. *Crop Sci*. 35: 889-894

Mandel, M. and Higa, A. 1970. Calcium dependent bacteriophage DNA infection. *J. Mol. Biol*. 53: 159-162

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

Mangin, B. 1999. Temporal and multiple quantitative trait loci analyses of resistance to bacterial wilt in tomato permit the resolution of linked loci. *Genet*. 151:1165–1172

Markose, B. L. 1996. Genetic and biochemical basis of resistance to bacterial wilt in chilli. M.Sc. (Ag) thesis, Kerala Agricultural University, Thrissur. 140p.

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

Mehdy, M. C. 1994. Active oxygen species in plant defense against pathogens. *Pl. Phys.* 105:467-472.

Messiaen, C. M. 1975. L'aubergine. In: Le Potager tropical, Cultures spéciales. Presses Universitaires de France, Coll. Techniques vivantes. pp. 231-249.

Mew, T.W. and Ho, W.C. 1976. Varietal resistance to bacterial wilt in tomato. *Pl. Dis. Rep*. 60: 264-268.

Miao, Z., Shou, S., Jiayan C., Jiang, F. Zhu, Z., and Li, H. 2007. Identification of two AFLP markers linked to bacterial wilt resistance in tomato and conversion two SCAR markers. *Mol. Bio. Rep*. 36:479-486.

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

Michelmore, R. 1995. Molecular approaches to manipulation of disease resistance genes. *Annu. Rev. Phytopathology*. 15: 393-427

Michelmore, R.W., Paran, I., and Kesseli, R.V. 1991. Identification of markers linked to disease resistance genes by bulk segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci*. USA. 88: 9828-9832.

Milla, S. R., Levin, J. S., Lewis, R. S., and Rufty, R. C. 2005. RAPD and SCAR markers linked to an introgressed gene conditioning resistance to *Peronospora tabacina* D.B. Adam. in tobacco. *Crop Sci*. 45: 2346-2354.

Minamiyama, Y., Kinoshita, S., Inaba, K., and Inoue, M. 2005. Development of a cleaved amplified polymorphic sequences (CAPS) marker linked to pungency in pepper. *Pl. Breed*. 124(3): 288-291

Mirshamsi, A., Farsi, M., Shahriari, F., and Nemati, H. 2008. Use of random amplified polymorphic DNA markers to estimate heterosis and combining ability in tomato hybrids. *Pakistan J. Biol. Sci*. 11(4).499-501.

Mochizuki, H, Yamakawa, K. 1979. Resistance of selected eggplant cultivars and related wild *Solanum* species to bacterial wilt (*Pseudomonas solanacearum*). Bull. Veg. Ornamental Crop Res. Stn. A. 6: p. 10.

Mohamed, M. E. S., Umaharan, P., and Phelps, R. H. 1997. Genetic nature of bacterial wilt in tomato accession: A1421. *Euphytica*. 96: 323-326

Mohan, M., Nair, S., Bentur, J. S., Rao, U. P., and Bennett, J. 1994. RFLP and RAPD mapping of the rice Gm^2 gene that confers resistance to biotype 1 to gall midge (*Orseolia oryzae*). *Theor. Appl. Genet*. 87: 782-788.

Monma, S. and Sakata, Y. 1983. Inheritance of resistance to bacterial wilt in tomato. *ACIAR Proc*. 45: 149-153.

Monma, S. and Sakata, Y. 1993. Inheritance of resistance to bacterial wilt in tomato. *ACIAR Proc*. 45: 149-153.

Monna, L., Miyao, A., Zhorg, M. S., Sasaki, T., and Minobi, Y. 1995. Screening of RAPD markers linked to photoperiod –sensitivity gene in rice chromosome 6 using bulk segregant analysis. *DNA Res*. 2: 101-106.

Moon, H. and Nicholson, J.S. 2007. Indentification of AFLP and SCAR Markers Linked to tomato spotted wilt virus resistance in tobacco. *Crop Sci.* 47: 1887-1894

Mullis, K. B. and Faloona, F. A. 1987. Specific synthesis of DNA *in vitro via* a polymerase chain reaction. *Methods in enzymology*. 225: 335-350

Mullis, K. B., Faloona, F. A., Scharf, S., Saiki, R., Horn, G., and Erlich, E. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbor Symp. *Quant*. *Biol.* 51: 263-273

Murayama, S., Habuchi, T., Yamagishi, H., and Terachi, T. 2003. Identification of a sequence-tagged site (STS) marker linked to a restorer gene for Ogura cytoplasmic male sterility in radish (*Raphanus sativus* L.) by non-radioactive AFLP analysis. *Euphytica.*129(1): 61-68.

Murray, H. G., Thompson, W. F. 1994. Rapid isolation of high molecular weight DNA. *Nucleic Acids Res.* 8:4321-4325

Murugaiyan, S., Bae, J. Y., Wu, J., Lee, S. D., Um, H. Y., Choi, H. K., Chung, E., Lee, J. H. and Lee, S. W. 2010. Characterization of filamentous bacteriophage PE226 infecting *Ralstonia solanacearum* strains. *J. Appl. Micrbiol.* 10:1365-2672.

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

Nakaho, K., Hibino, H., and Miyagawa, H. 2000. Possible mechanism limiting movement of *Ralstonia solanacearum* in resistant tomato tissues. *J. Phytopathology*. 148:181–190.

Naqvi, N. I. and Chattoo, B. B. 1995. Development of a sequence characterized amplified region (SCAR) based indirect selection method for a dominant blast resistance gene in rice. *Genome*. 39:26-30.

Narayanan, K. K. 1984. Evaluation of inter varietal hybrids of brinjal for yield and resistance to bacterial wilt. MSc (Ag) thesis. Kerala Agricultural University, Thrissur. 78p

Nedim, M., Filiz, H. B., Münevver, G., and KazÂm, A. 2008. Development of SRAP, SRAP-RGA, RAPD and SCAR markers linked with a Fusarium wil resistance gene in eggplant. *Theor. Appl. Genet*. 117: 1303-1312.

Nematzadeh, G. A., Huang, N., and Khush, G. S.2004. Mapping of the gene for aroma in Rice (*Oryza sativa*. L) by BSA via RAPD markers. *J. Agric. Sci .Technol*.6:129-137

Nirmal-Jyothi, A., Nagarajan, P., Rabindran, R., Shanmugasundram, P., and Senthil, N. K. 2001. RAPD markers linked to rice blast (*Magnaporthe grisea* Cav.) resistant gene. *Trop. Agric. Res.* 13: 373-379

Nouhi, A., Amiri, R., Haghnazari, A., Saba, J., and Misbah, M. 2008. Tagging of resistant gene (s) to rhizomania disease in sugar beet (*Beta vulgaris*). *Afr. J. Biotech*. 7(4): 430-433.

Nunome, T., Ishiguro, K., Yoshida, T., and Hirai, M. 2001. Mapping of fruit shape and color development traits in eggplant (*solanum melongana* l.) based on RAPD and AFLP markers. *Breed. Sci*.51:19-26.

Olson, L. J., Steven, M., Selmanp, T. B. R., and Keegstra, K. 1989. ATP Is Required for the Binding of Precursor Proteins to Chloroplasts. *J. Bio, Chem*. 264:6724-6729.

Ono, K., Hara, H., and Akazawa, J. 1984. Ecological studies on the bacterial wilt, caused by *Pseudomonas solanacearum*. V. The movement of the pathogen in tobacco plants. *Bulletin of the Okayama Tobacco Experiment Station* No. 43, pp. 41-46.

Onozaki, T., Tanikawa, N., Taneya, M., Kudo, K., Funayama, T., Ikeda, H., and Shibata, M. 2004. A RAPD-derived STS marker is linked to a bacterial wilt (*Burkholderia caryophylli*) resistance gene in carnation. *Euphytica*. 138: 255-262.

Opena, R. T. and Tschanz, A.T. 1987. Bacterial wilt resitance program on tomato at AVRDC. ACIAR bacterial Wilt Nwsl. (2):1-2.

Opena, R. T., Hartman, G. L. Chen, J. T., and Yang, C. H. 1990. Breeding for bacterial wilt resistance in tropical tomato. Proc Int Conf Plant Prot Trop 3rd Genting Highlands Pahang Malaysia. 44–50

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

Osiru, M. O., Rubaihayo, P. R., and Opio, A. F. 2001. Inheritance of resistance of tomato to bacterial wilt and its implication for tomato improvement in Uganda. *Afr. Crop Sci. J.* 9: 9-16.

Ouedrago, J., Tignegre, J. B., Timko, H. P., and Belzite, F. J. 2002. AFLP markers linked to resistance against *Striga gesnerioides* race 1 in cow pea (*Vigna unguiculata*). *Genome*. 45: 787-793.

Paran, I. and Michelmore, R. W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet*. 85: 985-993

Paterson, A. H., Lander, E. S., Hewitt, J. D., Peterson, S., Lincoln, E., and Tanksley, S. D. 1988. Resolution of quantitative traits into mendelian factors by using a complete linkage map of restriction fragment length polymorphism. *Nat*. 335-:721-726.

Pehu, R., Harry, S. K., and William, A. H. 2006. Analyses of Solanaceous species using repetitive genomic DNA sequences isolated from *Solanum* spp. *Pl. Sci.*117:121-127.

Petrolini, B., Quaroni, S., and Saracchi, M. 1986. Scanning electron microscopy investigations on the relationships between bacteria and plant tissues. II. Investigations on the initial process of *Pseudomonas solanacearum* pathogenesis. *Rivista di Patologia Vegetale* 22:100-115.

Pradhanang, P. M., Elphinstone, J. G., and Fox, R. T. V. 2000. Identification of crop and weed hosts of *Ralstonia solanacearum* biovar 2 in the hills of Nepal. *Pl. Path*. 49:403–413.

Prior, P. and Fegan, M. 2005. Recent developments in the phylogeny and classification of *Ralstonia solanacearum*. *Acta Hort*. 695: 127-136.

Procunier, J. D., Knox, R. E., Bernier, A. N., Grag, M. A., and Howes, N. K. 1997. DNA markers linked to a T10 loose smut resistance gene in wheat (*Triticum aestivum* L.). *Genome*. 40: 176-179

Quirin, E. A., Ogundiwin, E. A., Prince, J. P., Mazourek, M., Briggs, M. O., Chlonda, T. S., Kim, K. T., Falise, M., Kang, B.C., and Jahn, M. M. 2005. Development of sequence characterized amplified region

(SCAR) primers for the detection of *Phyto.5.2.*, a major QTL for resistance to *Phytophthora capsici* Lean in pepper. *Theor. Appl. Genet*. 110(4): 605-612

Ragina, V. C. 2009. Molecular markers for bacterial wilt resistance in mapping population of tomato.MSc (Ag) thesis. Kerala agricultural University,Thrissur.70p

Rajan, S. 1985. Selection efficiency and genetic and biochemical basis of resistance to bacterial wilt in tomato. Ph.D. (Ag) thesis. Kerala Agricultural University, Thrissur. 179p.

Rao, K. K. 2003. Molecular tagging of a new bacterial blight resistance gene in rice using RAPD and SSR markers. *Pt. Breed. Theor. Appl. Genet*. 17: 73-74.

Rashid, M. A., Rahman, M. A., Alam, S. N., Faruk, I., Jasmine, H. S., Sultan, N. A., Black, L., Wang, J. F., Talikar, N.S., Luther, G., and Miller, S. 2002. Varietal screening of eggplant for resistance to bacterial wilt, fruit and shoot borer, leaf hopper (jassids) and root knot nematode. *Bangladesh. J. Bot*. 39 (2):63-68

Rashmi Kumari 2008. Molecular characterization of chilli (*Capsicum annuum L.*) genotypes for tagging bacterial wilt resistance gene. Msc. (Ag) Thesis. Kerala Agricultural University, Thrissur . 150p.

Rifty, R. C., Yi, H. H., and Wernsman, A. 1998. Mapping the root –knot nematode resistance gene (Rk)in tobacco with RAPD marker. *Pl. Dis*. 82(12):1319-1322.

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

Ross, A. F. 1961. Systemic acquired resistance induced by localized virus infections in plants. *Virol.* 14:340-358.

Rowe, P. R., and Sequeira, L. 1970. Inheritance of resistance to *Pseudomonas solanacearum* in *Solanumphureja*. *Phytopathology*. 60:1499–1501.

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

Sajid-Ur–Rahman., Malik, T. A., Ashraf, M., and Ahsan, M. 2008. Identification of a DNA marker for nectariless trait in cotton using random amplified polymorphic DNA technique. *Pakistan. J. Bot*. 40(4): 1711-1719.

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

Sandhu, S. S., Bastos, C. R., Azini, L. E., Neto, A. T., and Colombo, C. 2002. RAPD analysis of herbicide resistant brasislian rice lines produced via mutagenesis. *Genet*. *Mol. Res.* 1(4): 359-370.

Schell, M. A. 2000. Control of virulence and pathogenicity gene of Ralstonia solanacearum by an elaborate sensory network. *Annu. Rev*. *Phytopathology*. 38:263–292

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

Sequeira, L. and Rowe, P.R. 1969. Selection and utilisation of *Solanumphureja* clones with high resistance to different strains of *Pseudomonas solanacearum*. *Am.Potato. J.* 46, pp.451–462.

Sheela, K. B. 1982. Cataloguining of Brinjal germplasm to isolate line(s) resistant to bacterial wilt. M.Sc (Ag) thesis, Kerala Agricultural University, Thrissur. 55p

Shen, Y. W., Guam, Z. Q., Lu, J., Zhuang, J. Y., Zheng, K. L., Gao, M. W., and Wang, X. M. 1998. Linkage analysis of a fertility restoring mutant generated form CMS rice. *Theor. Appl. Genet*. 97: 261-266

Shou, S. Y., Feng, Z. Z., Miao, L. X., and Liao, F. B. 2006. Identification of AFLP markers linked to bacterial wilt resistance gene in tomato. *Hereditas.* 28(2):195-199.

Singh, K. 1961. Inheritance of North Carolina type of bacterial wilt resistance in tomato lycopersion esculentum L (Master Thesis). University of Hawaii, Honolulu.

Singh, M., Choudhary, K., and Boss, K. S. 2006. RAPD based SCAR marker SCA12 linked to recessive gene conferring resistance to anthracnose in sorghum [*Sorghum Bicolor* (L). Moench]. *Theor. Appl. Genet*. 114: 187-192.

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

Smith E. F. 1896. A bacterial disease of the tomato, eggplant and Irish potato (*Bacillus solanacearum* nov. sp.).*U.S. Dept. Agric. Div. Veg. Phys. and Pathol. Bull.* 12:1-28

Sreelathakumary, I. 1983. Incorporation of two main sources of resistance to bacterial wilt in F1 generation of tomato. M.Sc. (Ag) thesis. Kerala Agricultural University, Thrissur. 81p.

Steven, M. R., Lamb, E. M., and Rhoads, D. D. 1995. Mapping the *Sw-5* locus for tomato spotted wilt virus resistance in tomatoes using RAPD and RFLP analyses. *Theor. Appl. Genet.* 91: 451-456.

Stiles, J. I., Lemme, C., Sondur, S., Morshidi, M. B. and Manshardt, R. 1993. Using randomly amplified polymorphic DNA markers and evaluating genetic relationships among papaya cultivars. *Theor. Appl. Genet*. 85: 697-70

Strider, D. L., Jones, R. K., and Haygood, R. A. 1981. Southern bacterial wilt of geranium caused by *Pseudomonas solanacearum*. *Pl. Dis.* 65:52–53.

Sudhamayee, M. 2010. Sex determination in nutmeg (*Myristica fragrans* Hott.) through molecular and biochemical markers. M.Sc. (Ag) thesis. Kerala Agricultural University. Thrissur. 120p.

Sugita, T., Yamaguchi, K., Sugimura, Y., Nagata, R., Yuji, K., Kinoshita, T., and Todozoki, A. 2004. Development of SCAR markers linked to L^3 gene in Capsicum. *Breed. Sci.* 54:111-115

Sun, B, Yi, L., Zhiliang, L., Zhenxing, L., Sun, G. 2008. AFLP Markers Linked to Genes Related to Bacterial Wilt Resistance of eggplant. *Mol. Pl. Breed.* 6:929-934.

Suzuki, I., Sughara, Y., Katani, A., Todaka, S., and Shimada, H. 1964. Studies on breeding eggplants and tomato for resistance to bacterial wilt. Investigation on method of evaluating the resistance

Melampsora medusa and on the source of resistance in eggplants and tomatoes. *Bull. Hort. Res. St. Hiratsuka. Scr. A*. 3:77-106.

Swaminathan, M. and Srinivasan, K. 1972. Studies on brinjal hybridization II.Transference of bacterial wilt resistance from wild brinjal variety. *Agric. Res. J. Kerala*. 9: 11-13.

Tabor, G. M., Kubisiak, T. L., Klopfenstein, N. B., Hall, R. B., and Mcnabb, H. S. 2000. Bulked segregant analysis identifies molecular markers linked to resistance in *Populus deltoids*. *Genetics*. 9:1039-1042.

Tai, T. H., Dahlbeck, D., Clark, E. T., Gajiwala, P., Pasion, R., Whalen, M. C., Stall, R. E., and Staskawicz, B. J. 1999. Expression of the *Bs2* pepper gene confers resistance to bacterial spot disease in tomato. *Proc. Natl. Acad. Sci.* USA 96, 14153–14158.

Tans-Kersten, J., Guan, Y. F., and Allen, C. 1998. *Ralstonia solanacearum* pectin methylesterase is required for growth on methylated pectin but not for bacterial wilt virulence. *Appl. Environ. Microbiol*. 64(12):4918–4923.

Techawongstien, S., Khemnak, S., and Theerakulpisut, P. 2006. Determination of the inheritance of pungency in *Capsicum* by molecular techniques. (On line): Available www.actahort.org (28 June 2008).

Thoquet, P., Olivier, J., Sperisen, C., Rogowsky, P., Prior, P., Anais, G., Mangin, B., Bazin, B., Nazer, R. and Grimsley, N. 1996. Polygenic resistance of tomato plants to bacterial wilt in the French West Indies. *Mol. Plant Microbe Interact*. 9:837–842.

Thurston, H. D. 1976. Resistance to bacterial wilt *(Pseudomonas solanacearum*). In: Sequeira L, Kelman A (eds) Proceeding of the first international planning conference and workshop on the ecology and control of bacterial wilt caused by *Pseudomonas solanacearum*. NC state university, Raleigh, pp. 58-62.

Tian, S. B., Wang, Y. Q., Luo, Z. Y., Li, M., Chen, L., Chen, Y. K., and Hong, Y. J. 2007. Genetic analysis of resistance to bacterial wilt in eggplant *(Solanum melongena*), Southwest China. *J. Agric. Sci.* 20: 642-645.

Tikkoo, S. K., Kishun, R., and Reddy, P. P. 1990. Breeding for combined resistance to bacterial wilt and rootnot nematode in tomato and pepper production in the tropics. S. K. Green, t. Griggs and B. T. Mclean (eds.). AVRDC. Tainan, Taiwan. p. p.99-106.

Tingley, S.V. and Tufo, D. 1993. Genetic analysis with random amplified polymorphic DNA markers. *Pl. Physiol.* 101: 349-352

Tuzun, S., and Kuc, J., 1991. Plant immunization: an alternative to pesticides for control of plant diseases in green house and "eld. In: Bay-Peterson, J. (Ed.), The Biological Control of Plant Diseases. Food and Fertilizer Technology Centre, Taiwan, pp. 30-40.

Van Loon, L. C., Gerritsen, Y. A. M., and Ritter, C. E. 1987. Identification, purification and characterisation of pathogenesis-related proteins from virusinfected Samsun NN tobacco leaves. *Pl. Mo. Biol*. 9:593-609.

Vanitha, S. C., Niranjana, S. R., Mortensen, C. N., and Umesha, S. 2009. Bacterial wilt of tomato in Karnataka and its management by *Pseudomonas fluorescens*. *BioControl*. 54:685–695.

Varghese, G. 1991. Evaluation of F1 hybrids resistant to bacterial wilt and inheritance of resistance in brinjal (*S. melongena* L.). M.Sc. (Ag) thesis. Kerala Agricultural University, Thrissur. 89p.

Varma, S. P. C. 1995. Variability and heterosis in green fruited brinjal. M.Sc (Ag) thesis .Kerala Agricultural University. Thrissur, 104p.

Virk, P. S., Newbury, H. J., Jackson, M.T., and Ford-Lloyd, B. V. 1995. The identification of duplicated accessions within a rice germplasm collection using RAPD analysis. *Theor. Appl. Genet*. 90: 1049-1055.

Wang, J. F. 1971. Basic protocols for conducting research on tomato bacterial wilt caused by *Pseudomonas solanacearum*. *AVRDC*. Taiwan. 23p

Wang, J. F., Olivier, J., Thoquet, P., Mangin, B., Sauviac, L., and Grimsley, N. 2000. Resistance of tomato line Hawaii7996 to Ralstonia solanacearum Pss4 in Taiwan is controlled mainly by a major strain-specific locus. *Mol. Pl. Microbe Interact*. 13:6–13.

Wang. S. W. Y., Fauquet, Z. L. H., and Ronald, P. 1995. A receptor kinase like protein encoded by the rice disease resistance gene Xa21. *Sci*. 270: 1804-1806.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Metraux, J. P., and Ryals, J. A. 1991.Coordinate gene activity in response to agents that induce systemic acquired resistance. *Pl. Cell*. 3:1085-1094.

Waugh, R. and Powell, W. 1992. Using RAPD marker for crop improvement. *Trends. Biotech*.10: 186-191.

Welsh, J., Hoenycutt, R. J., McClelland, M. and Sobral, B. W. S. 1991. Parentage determination in maize hybrids using arbitrarily primed polymerase chain reaction (AP-PCR). *Theor. Appl. Genet*. 82: 473-476

Wen, W. E., Le, G. Q., He, Z. H., Yang, W. Y., Xu, M. L., and Xi, X. C. 2008. Development of an STS marker tightly linked to *Yr26* against wheat stripe rust using the resistance gene-analog polymorphism (RGAP) technique. *Mol. Breed* . 22(4). 507-515.

Wenneker, M., Verdel, M. S. W., Van Beuningen, A. R., Derks, J. H. J., and Janse, J. D. 1999. *Ralstonia (Pseudomonas) solanacearum* race 3 (biovar 2) in surface water and natural weed hosts: first report on stinging nettle (*Urticadioica*). *Eur. J. Pl.Path*. 105:307–315.

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

Williams, M. N. V., Pandi, N., Nair, S., Mohan, M., and Bennett, J. 1991. Restriction fragment length polymorphism analysis of polymerase chain reaction products amplified from mapped loci of rice (*Oryza sativa* L.) genomic DNA.*Theor. Appl.Genet*. 82: 489-494.

Winstead, N. N. and Kelman, A. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. *Phytopathology*. 42: 628-634

Wongse, J., Bunn, T. B., Crossman, C., Jiang, J., and Tanksley, S. D. 1994. Chromosomal localization and molecular marker tagging of the powdery mildew resistance gene in tomato. *Theor. Appl. Genet*. 89:76-79.

Wyman, A. and White, R. 1980. A highly polymorphic locus human DNA. *Proc. Natl. Acad. Sci.* USA. 77:6754-6758.

Xiao, J., Li, J., Yuan, L., McCouch, S.R. and Tanskley, S.D. 1996. Genetic diversity and its relationship to hybrid performance and heterosis in rice as revealed by PCR based markers. *Theor. Appl. Genet*. 92: 637-643.

Xiao, L. Z., Zhu, Z. D., He, K. L., Zhou, M. N., and Lin, G. F. 1983. Observations of the infected portion of mulberry bacterial wilt by scanning electron microscopy. *Sci. Seric.* 9:58-59.

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., and Nishiuchi, Y. 1995. Transfer of two Burkholderia and an Alcaligenes species to Ralstonia gen. nov.: proposal of Ralstonia pickettii (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiol. Immunol.* 39, 897–904.

Yagi, M., Onzaki, T., Taneya, M., Watanable, H., Yoshimura, T., Ochiai, T. Y. Y., and Shibata, M. 2006. Construction of genetic linkage map for the Carnation by using RAPD & SSR marker and mapping quantitative trait loci (QTL) for resistance to bacterial wilt caused by Burkholderia caryphylli. *J. Jpn. Soc. Hortic. Sci.* 75 (2):166-172.

Yang, J. G., Pi, X. H., Chen, H. M., Yao, Y. G., Zou, Y. 2006. Resistance of ER300 eggplant (*Solanum melongena* L.) against genetic bacterial wilt *(Ralastonia solanacearum*) and it's application to breeding. J. Hunan Agric. Univ. (Natural Science Edition). 32(3): 277-279.

Yi, H. Y., Rufty, R. C., and Wernsman, E. A. 1998. Mapping the root-knot nematode resistance gene (*Rk*) in tobacco with RAPD markers. *Pl. Dis.* 82(12): 1319-1322.

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

Yu, K., Park, S. J., and Poysa, V. 1999. Abundance and variation of microsatellite DNA sequences in beans (*Phaseolus vigna*). *Genome.* 42: 27-34.

Yue, S. J., Wu, D. H., and Liang, C. Y. 1995. Studies on resistance heredity of bacterial wilt of tomato. *J. South China Agric. Univ*. 16: 91-95.

Yui, M., Monma, S., Hirai, M., Nishimura, S., Ukai, Y., and Enomoto, S. 1999. Random amplified polymorphic DNA (RAPD) markers for the selection of tomatoes resistant to bacterial wilt. Natl. Res. Inst. Vegetable Orna Mental Plants Tea Bll (Series A), 14: 189-198.

Zhang, F., Wang, G., Wang, M., Liu, X., Zhao, X., Yu, Y., Zhang, D., and Yu, S. 2008. Identification of SCAR markers linked to or, a gene inducing beta-carotene accumulation in Chinese cabbage. *Euphytica*. 831-839.

Zhang, G., Angeles, E. R., Abenes, M. L. P., Khush, G. S. and Huang, N. 1996. RAPD and RFLP mapping of the bacterial blight resistance gene *Xa-13* in rice. *Theor. Appl. Genet*. 93: 65-70.

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

Zhang, H. B., Li, W. Z., Chen, J., Yang, Y. H., Zhang, Z. J., Zhang, H. W., Wang, X. C. and Huang, R. F. 2007. Transcriptional activator TSRF1

reversely regulates pathogen resistance and osmotic stress tolerance in tobacco. *Pl. Mol. Biol*. 63(1):63–71.

Zhang, Q., Bharaj, T. S., Virmani, S.S., Huang, H., 1997. Mapping of the Rf-3 nuclear fertility restoring gene for WA cytoplasmic male sterility in rice using RAPD and RFLP markers. *Theor. Appl. Genet*. 94:27-33.

Zhu, H. W., Yao, Y. G., Liu, Z. M., Yang, J. G., Chen, H. M., and Zou, X. X. 2005. Studies on RAPD marker of bacterial wilt resistance gene in eggplant (*Solanum melongena*). *Acta Hotic. Sinica*. 32(2): 321-323.

Zhu, H. W., Yao, Y. G., Liu, Z. M., Yang. J. G., and Chen, H. M. 2004. On resistance to bacterial wilt in eggplant(*Solanum melongena*), J. Hunan Agric. Univ. (Natural Science Edition). 30(3): 288-289.

Zhu, L., Chne, Y., Ling, Z., Xu, Y. and Xu, J. 1993. Identification of molecular markers linked to blast resistance gene in rice. In: *You. Chen. Agric. Biotechnol*. China Science and Technology press, Beiging, China. pp 213- 225.

Appendices

APPENDIX- I

List of Laboratory Equipments used for the study

NanoDrop^{R} ND-1000 spectrophometer	Thermo Scientific, USA.
Cooling Centrifuge	KUBOTA model No. 65000, Japan
	MIKRO 22R Heittich Zentrifugen U
Minispin	Eppendorf, Germany
Horizontal electrophoresis systems	BIO-RAD, Italy and GeNei India
Thermal cycler	Mastercycler and mastercycler
	Gradient, Eppendorf, Germany.
Gel documentation system	BIO-RAD Inc. Italy.
AccuBLOCK ^{TM} Digital Dry Bath	model D1100, Labnet international, Inc
	USA
Laminar air flow	THERMODYNE, Faridabad, Haryana;
	Rotex, B and C Industries, Kerala

APPENDIX- II

Composition of reagents used for DNA isolation and purification

Roger and bendich (CTAB) method

I. 1 X CTAB extraction buffer :

a. CTAB (1% V/V)
b. 100mM Tris buffer (pH 8)
c. 20mM EDTA (pH 8)
d. 1.4 M NaCl

II. 10% CTAB solution :

a. 10% CTAB (w/v)
b. 0.7 M NaCl

III. TE Buffer :

a. 10mM Tris buffer (pH 8)- 1ml
b. 10mM EDTA (pH 8)-0.4 ml

Distilled water - 98.6 ml

IV. 76% washing solution :

a. Ethanol 76%
b. 10 mM Ammonium acetate

To 76 parts of absolute ethanol, 24 parts of double distilled water and 1ml of 10 mM Ammonium acetate was added.

V. Chilled isopropanol

Vi. RNase A stock

RNase A	-	10 mg
Distilled water	-	1 ml

Stock was prepared by dissolving 10 mg RNase A in 1 ml water and was stored under refrigerated conditions at -20^{0}C. The RNase A was used to prepare RNase. Ten per cent RNase solution was prepared by dissolving the same in water at 1:10 ratio. The solution was stored at -20^{0}C and use for RNase treatment.

APPENDIX- III

Composition of Buffers and Dyes used for gel electrophoresis

I. TAE Buffer 50X (for 1 litre):

242 gram Tris buffer

57.1 ml glacial acetic acid

100 ml 0.5 EDTA (pH 8)

The solution was prepared and stored at room temperature

II. Loading dye (6X):

0.25% Bromophenol Blue

0.25%Xylene Cynol

30% Glycerol in water

The dye was prepared and kept in fridge at 4^0C

III. Ethidium bromide (intercalating dye)

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

APPENDIX-IV

Composition of different reagents, media and master mix used for Cloning and Transformation studies

I. Solution A: Competent cell preparation (Genie, Bangalore)

a. Ice-cold 100Mm $CaCl_2$

II. Luria Bertani (LB) broth:

a.	Tryptone	10 gm.
b.	Yeast extract	5 gm.
c.	NaCl	5 gm.
d.	pH adjusted to 7	
e.	Distilled water	1 lit.

III. Luria Bertani Agar medium (LBA):

a.	Tryptone	10 gm.
b.	Yeast extract	5 gm.
c.	NaCl	5 gm.
d.	Agar	20gm.
e.	pH adjusted to 7	
f.	Distilled water	1 lit.

IV. Ampicillin 100 mg/ml

V. Preparation of master mix

i.	IPTG	6 µl (Stock 200mg/ml in water, working 50 µg/ml)
ii.	X-gal	12 µl (Stock 100mg/ml in DMSO, working 80 µg/ml)
	Autoclaved water	25 µl
Total	=	43 µl/ Petri plate

TAGGING OF BACTERIAL WILT RESISTANCE GENE IN *SOLANUM MELONGENA* VAR. *INSANUM* BY MOLECULAR MARKERS

By

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ABSTRACT OF THE THESIS

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