IDENTIFICATION OF AFLP MARKER LINKED WITH BACTERIAL WILT RESISTANCE IN CHILLI
(*Capsicum annuum* L.)

By

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(2011-11-109)

THESIS

Submitted in partial fulfillment of the requirement

for the degree of

Master of Science in Agriculture
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CENTRE FOR PLANT BIOTECHNCLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2013

DECLARATION

I, hereby declare that this thesis entitled **"Identification of AFLP marker linked with bacterial wilt resistance in Chilli (*Capsicum annuum* L.)"**is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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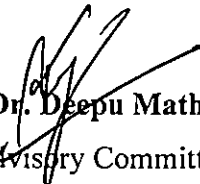
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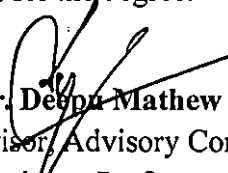
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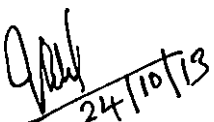Centre for Plant Biotechnology and Molecular Biology (CPBMB)
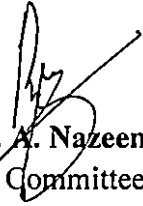
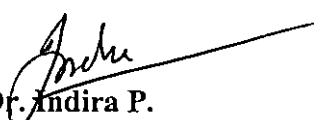College of Horticulture

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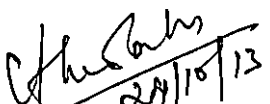
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ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AVRDC	Asian Vegetable Research and Development Centre
BSA	Bulk Segregant Analysis
BW	Bacterial wilt
bp	Base pair
β	Beta
C	Cytosine
CAPS	Cleaved amplified polymorphic sequences
CPBMB	Centre for Plant Biotechnology and Molecular biology
CTAB	Cetyl Trimethyl Ammonium Bromide
^{0}C	Degree Celsius
cm	Centimeter
Cm	Centi Morgan
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene Diamine TetraAcetic Acid
FYM	Farm yard manure
g	Gram
G	Guanine
hr	Hour (s)
HCl	Hydrochloric Acid
IIHR	Indian Institute Horticultural Research
KAU	Kerala Agricultural University
Kb	Kilo basepairs
L	Litre
M	Molar
mA	Milli Ampere

MAS	Marker-Assisted Selection
Mb	Mega base pairs
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minutes
ml	Millilitre
Mm	Milli mole
μl	Micro litre
μM	Micro molar
μg	Microgram
μl	Microlitre
NaCl	Sodium Chloride
NIL	Near isogenic line
NTSyS	Numerical Taxomomy System of Multivariate Statistical Program
ng/μl	Nanogram per micro litre
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
%	Percentage
PVP	Poly vinyl pyrrolidone
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RB	Resistant bulk
RNA	Ribo Nucleic acid
RNase	Ribonuclease
RFLP	Restriction Fragment Length Polymorphism

RILs	Recombinant inbreed lines
RP	Resistant parent
S	Susceptible
sec	Second (s)
SB	Susceptible bulk
SCAR	Sequence Characterized Amplified Region
SNP	Single Nucleotide Polymorphism
SP	Susceptible parent
SSR	Simple Sequence Repeats
STS	Sequence tagged sites
T	Thymine
TAE	Tris Acetate EDTA
TE	Tris EDTA
TEMED	N, N, N, N- Tetramethylene ethylene diamine
TZC	Triphenyl Tetrazolium Chloride
U	Unit
UV	Ultra violet
V	Volts
v/v	Volume by Volume
w/v	Weight by Volume

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UV	Ultra violet
V	Volts
v/v	Volume by Volume
w/v	Weight by Volume

Dedicated to my beloved parents shri. Prabhakar Thakur and smt. Padamavati Thakur

Introduction

1. INTRODUCTION

Bacterial wilt (BW) incited by the soil-borne, vascular pathogen *Ralstonia solanacearum* (*Pseudomonas solanacearum*) (Smith, 1896; Yabuuchi *et al.,* 1992) is highly challenging and one of the most destructive diseases of solanaceous crops worldwide (Hayward, 1964; Poussier *et al.,* 1999). The disease is predominant in warm humid tropical and temperate regions of the world (Hayward, 1991). Kerala being characterized by warm, humid tropical climate and acidic soils is a hot spot for bacterial wilt disease. This bacterial pathogen grows either in the xylem or phloem tissues and interferes with the transport of water and nutrients in the plant. In susceptible varieties, yield losses up to 100 per cent are observed.

At the subspecies level, the pathogen was grouped by Buddenhagen *et al.,* (1962) into three races based on their ability to infect different plant species. Race 1 has a very wide host range and known as the Solanaceous race, race 2 as the *Musa* race and race 3 as the potato race. Bacterial wilt causes significant damage on many important crops like chilli, tomato, potato, brinjal, groundnut, ginger etc. under disease-favorable weather conditions. Now it is prevalent in different parts of India and world. In India the incidence of bacterial wilt of chilli was reported by Kelman (1953) and they observed that the causal organism of this disease is endemic in India throught out the west coast, central and Deccan plateau of Karnataka, western Maharashtra and Madhya Pradesh, eastern plains of Assam, West Bengal, Orissa and Chotta Nagpur.

During 2010-11, India produced 0.8 million tonnes of dry chilli from an area of 0.93 million hectares. Andhra Pradesh (30 per cent) is the largest producer of chilli in India followed by Karnataka (20 per cent), Maharashtra (10 per cent), Orissa (9 per cent), Tamil Nadu (8 per cent) and other states contributing 18 per cent to the total area under chilli. The total export of chilli from India during 2009-10 was 0.2 million tonnes valued Rs.1.3 billion rupees.

In the year 2008-09 under review Kerala exported 23525.85 tonnes of pepper valued at Rs.38543.29 lakhs. But now bacterial wilt is a major contributor to lower productivity of chilli, inflicting 30 per cent yield loss in susceptible varieties under mild condition and 100 per cent under severe infection (Kumar *et al.* 2004).

The host plant resistance has been the major strategy for managing bacterial wilt. One of the ways to develop varieties with durable rust resistance is to pyramid the genes for resistance in a single variety, using marker assisted selection (MAS) with molecular marker and for this, tagging of genes with molecular markers is the pre-requisite (Keen, 1999; Tripathi *et al.*, 2003; Huang and Roder, 2004).

In AFLP (Amplified Fragment Length Polymorphism) assay, total genomic DNA is digested using two restriction enzymes. Double-stranded nucleotide adapters are ligated to the DNA fragments to serve as primer binding sites for PCR amplification. Primers complementary to the adapter and restriction site sequence, with additional nucleotides at the 3'end, are used as selective agents to amplify a subset of ligated fragments (Vos *et al.*, 1995). Capillary electrophoresis is a high-throughput electrophoresis strategy which is powerful enough to detect even 30bp variation in the nucleotide sequences. This technique has been extensively used with plant DNA for the development of high-resolution genetic maps and for the positional cloning of genes of interest (Blears *et al.*, 1998). Thus AFLP combined with capillary electrophoresis could be used as a powerful tool to detect any polymorphism among the accessions, for the character under study.

Kerala Agricultural University has developed bacterial wilt resistant varieties in chilli and among them, Anugraha is leading one (Markose, 1996). Anugraha is hybrid derivative of the cross Ujwala x Pusa Jwala. Ujwala was used as donor parent for resistance (Gopalakrishnan and Peter, 1991). Pusa Jwala and

Anugraha are near isogenic lines (NILs) differing only in their response to bacterial wilt. Gene imparting bacterial wilt resistance in this variety is reported to be monogenic and incompletely dominant (Markose, 1996).

By considering the potential of MAS, attempts were previously made to identify the molecular markers linked tightly with the resistance gene and were not successful (Kumari, 2008). The present study was designed to identify the tightly linked AFLP markers for bacterial wilt resistance. For this, Bulk Segregant Analysis (BSA) (Michelmore *et al.,* 1991) using the DNA from resistant variety Anugraha, susceptible variety PusaJwala, donor for the resistance Ujwala and separately bulked DNA of susceptible as well as resistant F_2 plants from the cross Anugraha x Pusa Jwala was employed.

The objective of the study was 'to identify the AFLP marker linked with bacterial wilt resistance in chilli (*Capsicum annuum* L.)'.

Review of Literature

2. REVIEW OF LITERATURE

Chilli or hot pepper (*Capsicum annuum* L.) is one of the most important Solanaceous vegetable crops grown worldwide. It is grown throughout the world and year round in tropical and sub-tropical regions. Among the diseases of chilli, bacterial wilt is one of the devastating diseases and it severely hampers the cultivation. Recently, this disease caused by *Ralstonia solanacearum* (Smith, 1896; Yabuuchi *et al.* (1992) has risen to alarming proportion in the plains of India and has become one of the limiting factors of late, various varieties, modern technologies and voluminous literature have been generated towards the management aspect of bacterial wilt in this crop. In Kerala, losses in hot peppers, eggplants and tomatoes reach as high as 100 per cent (Mathew and Peter, 2004). Numerous efforts are going on to breed high yielding chilli varieties which are resistant to bacterial wilt. A reproducible molecular marker system that can detect the presence of gene offering resistance to bacterial wilt will be of at most useful in these breeding efforts. Apart from reducing the length of breeding cycle, molecular markers allow screening of segregating population without maintaining virulent bacterial isolates and with no influence of the environment.

2.1. Bacterial wilt (BW) disease in chilli

Over one hundred years have elapsed since Smith (1896) published the first description of *Pseudomonas solanacearum* which causes a wilt disease of Solanaceous plants. *Ralstonia solanacearum* (Yabuuchi *et al.*, 1992, Getachew *et al.*, 2011) is a soil borne bacterium that causes a serious disease known as bacterial wilt and sequencing information of 16s rRNA genes and polyphasic taxonomy led to the proposal of genus *Ralstonia* and the pathogen was renamed as *Ralstonia solanacearum*. It has a large host range of more than 200 species in 50 families (Aliye *et al.,* 2008). At the subspecies level, the pathogen was divided into three races (Buddenhagen *et al.*, 1962). Race 1 has a very wide host range and is known as the Solanaceous race, race 2 as the *Musa* race, and race 3 as the potato race. Bacterial wilt of pepper is caused predominantly by biovars 1

and 3 of *R. Solanacearum*, since they belong to race 1. Strains of *R. solanacearum* have been classified into five biovars (Kumar *et al.,* 2009) and five races (Li *et al.,* 2010; Buddenhagen *et al.,* 1962).

Wilting caused by this pathogen is an enormous risk for plants in tropical and sub-tropical climatic regions (Hayward, 1991; Milling *et al.,* 2011). Shekhawat *et al.* (1992) observed the endemic causal organism throughout the west coast, central and Deccan plateau of Karnataka, Western Maharashtra and Madhya Pradesh, Eastern plains of Assam, West Bengal, Orissa and Chotta Nagpur plateau on tomato, brinjal and chilli. Yabuuchi *et al.* (1992) transferred several species of rRNA homology group II *Pseudomonas solanacearum* to the genus *Burkholdia.* James *et al.* (2003) reported bacterial wilt disease was caused by biovar 1 and race 3 strains of *R. Solanacearum* by in-*vitro* culture condition using RAPD analysis.

Race differentiation was done based on host range and hypersensitivity reaction (HR) on capsicum by leaf infiltration technique used by Lozano and Sequeira (1970). The bacterial suspensions (OD 0.3 at 600 nm) were infiltrated into intercostal region on the under surface of capsicum leaves using a disposable syringe with slits needle removed. The barrel of the syringe was placed in close opposition to the under surface of the leaf and the pressure was gently applied. The plants were then examined at 14, 24, 48, 72 hours, up to 10 days.

SMSA culture medium, IFAS indicator plant or BOX-PCR methods were also used for proper identification of *R. Solanacearum* races and biovars (Galal *et al.,* 2003; Stefani *et al.,* 2005).

2.2. Strategies for managing bacterial wilt

Like other soil borne diseases, various strategies have been developed for managing bacterial wilt. But the control of bacterial wilt was proved to be very

difficult, especially for race1 with its broad host range. Methods to manage *R. solanacearum* are phytosanitation, cultural practices, chemical management, biocontrol and host resistance (Martin and French, 1985; Champoiseau *et al.*, 2010; Muthoni *et al.*, 2012).

The most widely accepted and promising strategy is breeding resistant cultivar or grafting plant on a resistant root stock. Many a times, resistance of the root stocks may be unstable (Hayward, 1991) and the scion was found to be latently infected with the pathogen (Nakaho *et al.*, 2000). The disease also can be controlled by application of fertilizers and cost-effective soil amendments (Schonfeld *et al.*, 2003 and Gorissen *et al.*, 2004) such as compost, manure, urea, calcium oxide. Murakoshi and Takahashi (1984) reported that attempts for chemical control with soil fumigants, which are hazardous to human health and the global environment, will have either slight or no effects.

Wang *et al.* (2010) inoculated *R. solanacearum* at seedling stage of tobacco and then treated with K_1 and K_2 antibacterial agents at serial concentrations and the result indicated that K_1 and K_2 can inhibit *R. solanacearum* growth entirely, at the concentration range from 1/50 to 1/5000. Wu *et al.* (2012) reported that the hydrazone derivatives containing a pyridine moiety produced by the reaction of intermediates 2 with different aldehydes in ethanol using 2-chloronicotinic acid and 2-amino-5-chloro-3-methyl benzoic acid could inhibit the growth of *Ralstonia solanacearum*.

Plant Growth Promoting Bacteria (PGPR) strains and *Trichoderma sp.* were reported to be a promising bio-control agent to control *Ralstonia solanacearum*. It was found that they were able to reduce the disease in different levels and increased the yield of tomato (Guo *et al.*, 2004; Paul *et al.*, 2012). Murthy and Shrinivas (2012) isolated and characterized bacterial wilt pathogen from various agro-climatic regions and screened for potential antagonists for its bio-control. Nguyen and Ranamukhaarachchi *et al.*, (2010) isolated and

evaluated potential soil-borne antagonists for suppression of growth of pathogen *R. solanacearum* under *in vitro* and *in vivo* conditions. Lwin and Ranamukhaarachchi (2006) reported seven antagonist isolates *viz.*, TR2, TR6, TR7, TR10, TR12, TR15, and LR10. Three antagonists, TR6, TR12 and LR10, that appeared to be most effective in *R. solanacearum* suppression under *in vitro* and *in vivo* conditions, were identified at the genus level analyzing 16S rDNA (BIOTEC Culture Collection, 2009). The isolates which produced 2, 4-diacetyl phloroglucinol (DAPG) metabolites inhibited the pathogen both under *in vitro* and green house conditions (Yuan *et al.,* 1998, Ramesh *et al.,* 2009).

The transgenic approach shows potential for the genetic improvement of the crop using a wide set of transgenes (Tripathi *et al.,* 2004). Various genetic strategies available for reducing the yield losses from bacterial wilt include identification of genes for resistance from the primary gene pool, molecular marker-aided selection, and fine mapping and cloning of genes for resistance, introduction of cloned genes through transformation into elite cultivars under tissue-specific expression (Lee *et al.,* 2003).

Tripathi *et al.* (2004) incorporated genes for antimicrobial peptides like Magainins, Cecropins, Attacins and Thionins into banana which have shown to be resistant to bacterial wilt disease.

2.3. Incidence and symptoms of pathogen

Incidence of pathogen hampered through the infection of roots of susceptible plant through wounds (Pradhanang *et al.,* 2005). Temperature is an important environmental factor that affects multiple plant pathosystems and their interactions with their hosts (Hayward, 1991). High ambient temperatures have been shown to induce bacterial wilt incidence at a faster rate than moderate temperatures (Ciampi and Sequeira, 1980).

Carter (1991) observed various disease symptoms viz., leaf dropping, browning of root and lower portion of stem, root rot due to infection from secondary bacteria, leaf epinasty, adventitious roots growing in the stems, and narrow dark stripes corresponding to the infected vascular bundles beneath the epidermis. The proportion of the incidence of this disease increased alarmingly due to the crop rotation with other Solanaceous alternate hosts of the causal organism. Unfortunately, varietal resistance is known to fluctuate both geographically and over time due to genetic variability of strains of the pathogens as well as the difference of the microclimates (Ajjappalavara, 2006). He has reported incidence of bacterial wilt is through the effect of mutagens on the brinjal local cultivar (Malapur) and impact of soil amendment and crop rotation with non-host. Slimy viscous ooze typically appeared on transverse- sectioned stems at the point corresponding to the vascular bundle. As a result, collapse and death of the plant took place by degradation of occluded xylem vessels and destruction of surrounding tissues (Smith, 1920; Kelman, 1953).

2.4. Methods for artificial bacterial inoculation

2.4.1 Preparation of inoculum

Suspensions of bacterial cells were made by washing 48 hours old colonies of plates containing TtcA (protein) into 100ml of sterile distilled water. This was further used to prepare dilutions of 1:1, 1:2, 1:4, 1:8, and 1:16. Percentage transmittances of each dilution were determined using a Bosch photo colorimeter at a wavelength of 600nm (Paul *et al.,* 2012).

2.4.2 Inoculation

Several inoculation techniques have been used to evaluate resistance level of plants. Two bacterial inoculation methods, leaf cutting and pricking were found to be effective for bacterial blight disease resistance scoring in transgenic rice plants containing *Xa21* gene (Hoque and Mansfield, 2005).

Chellemi *et al.* (1992) reported infested soil technique in which soil was collected from the base of plants with symptoms of bacterial wilt and inoculum density of the soil was determined by serial dilution plating technique. Seedlings were transplanted into the infested soil and after 42 days, symptomatic plants confirmed the presence of the pathogen.

Winsted and Kelman (1952) inoculated four-week-old seedlings with a sharp needle into the stem through a drop of bacterial suspension using the stem-puncture technique. Stem puncture technique was found to be effective in tomato to induce 100 per cent wilt incidence (Karmannil, 2007; Ragina, 2009).

In pin prick method, sterile pins dipped in inoculums were used to prick the leaves on sides and in the centre of rice seedlings (Di *et al.*, 1991). In rice, a comparison of inoculation methods showed that pin prick method produced highest infection over the clipping and paint brush methods for bacterial leaf blight disease *(Xanthomonas campestris pv. oryzae)* (Jabeen, 2011).

2.5. Mechanism of bacterial wilt disease

Walker (1952) reported that the pathogen first enters into the intercellular space of cortex from there it moves to pith and xylem vessels and wilting of plant is due to vascular plugging. Bacteria multiply rapidly in the xylem and form slime in abundance which cause a marked increase in the viscosity of vascular stream and they interfere with water movement resulting in wilting (Kelman, 1953). In the presence of host plant cells, a regulatory cascade activates secretion of cell wall-degrading enzymes and the cluster of hypersensitive reaction and pathogenicity genes encoding components of a type III secretion system. Once in the plant tissues, high densities of the pathogen increase expression of virulence genes and production of exo-polysaccharide which determine pathogenicity and bacterium can persist in the environment through diverse survival forms until contact with a new host (Alvarez *et al.*, 2008).

Infested soil is the main source of inoculums. The pathogen can survive in the rhizosphere of non-host plants, including weeds (Granada and Sequeira, 1993). Soil factors influence the survival of the bacterium. *R. solanacearum* can infect undisturbed roots of susceptible hosts through microscopic wounds caused by the emergence of lateral roots. Transplanting, nematodes, insects, and agricultural equipment are also able to wound roots. Bacteria then colonize the cortex and advance towards the xylem vessel, from where it rapidly spreads in the plant (Momol *et al.*, 2008).

Markose, (1996) reported studies on biochemical basis of resistance revealed that total phenol, OD phenol and protein content and enzyme activities had a positive association with bacterial wilt resistance. Ujwala, the resistant variety showed high protein content and had higher activities of peroxidase and polyphenol oxidase enzymes in all plant parts. The resistant variety had well developed secondary xylem with thick and compact piliferous layers and cortical cells compared to the susceptible variety.

In chilli plant system, the entry, movement, multiplication, colonization and infection of *R. solanacearum* was studied using radiotracer technique. The bacterial wilt resistant variety Ujwala and the susceptible variety Pusa Jwala were used to determine the pattern of distribution of bacteria in the host tissues. Sixty days old chilli seedlings were used for inoculation and the radioactivity of ^{32}P was taken as evidence for presence of bacteria in a particular tissue. The radio assay indicated that the resistant and susceptible genotypes differed in the accumulation of radiolabelled bacteria in different plant parts. The radiolabelled bacterial counts in both stem as well as leaves were found lesser in Ujwala than Pusa Jwala, after incubation for 24 hrs (Markose *et al.*, 2001).

2.6. Source of resistance

In India, resistance to bacterial wilt was assayed in the cross between susceptible Pusa Jwala and the resistant PBC384. Parental, cross and back cross populations were provided by AVRDC, and screened at IIHR using the local *Ralstonia solanacearum* isolate "Kerala RS". Resistance was not fully dominant, and the selected segregants were recommended to be re-evaluated and reselected once or twice to stabilize the resistance (AVRDC, 2009).

Pepper lines resistant to bacterial wilt have been reported from several laboratories. MC4 and MC5 are reported to be resistant for bacterial wilt (Matos *et al.*, 1990). In a preliminary report, a Malaysian accession, LS2341, was found to be resistant in Japan (Mimura, 2000). Matsunaga and Monma (1999) reported chilli cultivars Mie-midori, Ishii-midori and Akashi to be resistant to *R. solanacearum*. Mimura and Yoshikawa (2009) indicated that accession LS2341 had the highest level of resistance to all the strains of *R. solanacearum* examined. Genetic analysis of resistance in pepper was performed in the doubled haploid progeny from a cross between a resistant parental line PM 687 and a susceptible cultivar Yolo Wonder. From this study, it was also reported that the susceptibility to *tobacco mosaic virus* (TMV) and nematodes (*Meloidogyne* spp.) was significantly linked with resistance to bacterial wilt (Lafortune *et al.*, 2005).

Peter *et al.* (1992) evaluated four Indian hot chilli cultivars Pant C-1, KAU cluster, White Khandari and Chuna along with six US cultivars for reaction to nine isolates of *R. solanaecearum* (race1 and race3) and found that Pant C-1 was the most resistant one. Pious and Peter (1985) also observed resistance to bacterial wilt in KAU cluster. Goth *et al.* (1983) reported that KAU cluster (Manjari) was resistant to four race 1 isolates and one race 3 isolates of *R. solanacearum*.

Gopalakrishnan (1991), from his studies on resistance to bacterial wilt and inheritance of clusterness in chilli concluded that the accession, CA 33 which was further improved to the status of a variety "Manjari" was resistant to bacterial wilt disease. Gopalakrishnan and Peter (1991) studied 146 accessions of capsicum species for resistance to bacterial wilt and found that CA 219 (Ujwala) and CA 33 (Manjari) were highly resistant with good dry chilli yeilds..

Markose *et al.* (2001) has crossed the susceptible variety Pusa Jwala with resistant Ujwala, followed by two back crosses using susceptible variety as recurrent parent and advanced the F_1 to BC_2F_7. From this study, it was reported that the inheritance of resistance to bacterial wilt was monogenic and incompletely dominant. She has developed a hybrid derivative by advanced selection from the cross Ujwala x Pusa Jwala, named Anugraha, which is resistant to bacterial wilt and near isogenic line (NIL) of Pusa Jwala (Gokulapalan *et al.*, 2004).

2.7. R gene mediated resistance

R gene confer resistance to completely unrelated taxonomic groups like bacteria, fungi, viruses and nematodes by the activation of a defense response that prevents the pathogen from spreading (Bendahmane *et al.*, 1999; Vossen *et al.*, 2000). Grube *et al.* (2000) showed that R gene loci in the Solanaceous crop genera tomato, potato and pepper are present on corresponding positions and that homologs of previously identified R genes derived from tomato and tobacco could be identified in syntenious positions in pepper.

Specific recognitions between pathogen *Avr* and plant R proteins are crucial for the set of the resistance response and determine the issue of many plant–pathogen interactions by triggering plant defence. Disease results from the inactivation or absence of one or both partners (Flor, 1997). It has been postulated that R gene products are receptors for pathogen-encoded *Avr*

components (Baker *et al.*, 1997). *Pichiaguillier mondii* strain *R13*, a yeast isolated from Thai rambutan, has been shown to suppress *Colletotrichum capsici* in harvested chilli by enhancing the activities of phenylalanine ammonia-lyase, chitinase, and β-1,3-glucanase and the accumulation of capsidiol phytoalexin (Nantawanit *et al.*, 2010).

Avr bacterial proteins expressed in plants carrying the cognate *R* protein generally induce a cell death program (Nimchuk *et al.*, 2001) termed the hypersensitive response (HR), closely linked to resistance. They can also cause disease- like symptoms when expressed in plants lacking the appropriate *R* protein. According to 'guard model' (Dangl and Jones, 2001), in resistant host, the plant target that interacts with both R and *Avr* proteins is guarded by the *R* protein, preventing its manipulation by pathogen effectors. The recent characterization of *RIN4*, a negative regulator of plant defence, strengthens this model (Mackey *et al.*, 2002).

The *Arabidopsis thaliana RRS1* R gene confers broad-spectrum resistance to several strains of *R. solanacearum* (Deslandes *et al.*, 1998; Deslandes *et al.*, 2002). Although genetically defined as recessive, this R gene behaves as a dominant gene in transgenic plants. Several lines of evidence are in favour of *RRS1* R. In R gene signalling components, protein-protein interaction is essential for plant viability. However, genetic and biochemical evidence strongly suggests that *RIN4* is involved in pathogen recognition of RPM1-mediated resistance in *Arabidopsis thaliana* (Mackey, *et al.*, 2002). In many cases, a single R gene can provide complete resistance to one or more strains of particular pathogen, when transferred to a previously susceptible plant of the same species.

R genes that recognize virulence factors that are essential for the pathogen to cause disease. These *R* genes enable plants to recognize specific races of a pathogen and mount effective defense responses (hypersensitive response), increasing expression of defense-related genes, and production of anti-microbial

compounds, lignin formation, and oxidative burst in many plant-pathogen interactions. Recombination events of plant *R* genes give plants a selective advantage against rapidly evolving pathogen populations (Baker *et al.*, 1997). There are also prospects for transgenic use of single *R* genes that have previously been proven durable. For example, the pepper gene *Bs2* has provided long-standing resistance against bacterial spot disease caused by *Xanthomonas campestris* (Tai *et al.*, 1999).

2.8. Molecular markers for tagging and mapping the disease resistance

Molecular markers are commonly used to characterize genetic diversity within or between populations or groups of individuals because they typically detect high levels of polymorphism. Recently, genetic studies in host plant resistance hold promise to trace genes conferring resistance to bacterial pathogens.

Analysis of RAPD and SSR markers were evaluated somatic amphiploid hybrid clone for resistance to bacterial wilt by using race 1 and race 3 strains of *Ralstonia solanacearum*, originating from Reunion Island (Fock *et al.*, 2000). RAPD molecular marker linked to a bacterial wilt-resistance gene of eggplant and chilli were screened by the bulked segregant analysis (BSA) method and sequence characterized amplified region (SCAR) marker linked to bacterial wilt-resistance gene was also obtained (Hao *et al.*, 2009). James *et al.* (2003) tagged RAPD marker OPF 8 to the resistance to *R. solanacearum* race 3 in Solanaceous vegetables.

Diversity of *R. solanacearum* causing bacterial wilt of ginger and other hosts in India was also analysed using REP– PCR and RFLP–PCR which confirmed their host origin that isolate biovar 3 (Kumar *et al.*, 2004). Cook *et al.*, (1989) have assessed the diversity of the pathogen according to RFLP using hypersensitive response and pathogenicity (*hrp*) genes as probes.

2.8.1. Amplified Fragment Length Polymorphism (AFLP)

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos *et al.*, 1995). In AFLP, total genomic DNA is digested with two restriction enzymes and adaptors of known sequence are then ligated to the DNA fragments. Primers complementary to the adaptors, with additional 1-3 selective nucleotides on the 3' end, are used to amplify the restriction fragments. The PCR amplified fragments can then be separated by gel electrophoresis and the banding patterns visualized. AFLP profiles require no prior DNA sequence information and the number and nature of amplified fragments are altered by the choice of primer pair. The technique also has the advantage of sampling many loci simultaneously and in addition, it is more robust than arbitrary priming techniques such as RAPD, because more stringent conditions are used in the PCR.

AFLP provides a novel and very powerful for gene tagging technique of any origin or complexity (Vos *et al.*, 1995; Blears *et al.*, 1998). It is typically inherited in Mendelian fashion and may therefore be used for typing, identification of molecular marker, and mapping of genetic loci. Wang *et al.* (2005) evaluate genetic diversity of wild and cultivated pawpaw through AFLP and estimated high level of genetic diversity within population which indicated that conservation strategy should focus on preserving genetic resources in major population.

Based on the introduction of AFLP technique, the research advances in many crops were reviewed, including genetic tagging, genetic diversity detection, variety taxonomy, variety identification and pedigree analysis (Mathew, 2006).

PCR-based methodologies provide an alternative method for isolation of microsatellite loci. Microsatellite loci have been developed using an AFLP-PCR approach called FIASCO (Fast Isolation by AFLP of Sequences Containing repeats) (Zane *et al.*, 2002; Sun *et al.*, 2008; Zang *et al.*, 2008). The ability of AFLPs to distinguish among genotypes is not hindered by their bi-allelic nature

(presence or absence) and thus polymorphisms can be identified between very closely related genotypes.

AFLP markers have been used in mapping plant genomes such as barley (Becker *et al.*, 1995), tomato (Saliba-Colombani *et al.*, 2000), chromosome landing (Cnops *et al.*, 1996) and positional cloning (Simons *et al.*, 1998). Paran *et al.* (1998) evaluated the level of variation in *C. annuum* using different marker systems and the percentage of polymorphic markers was high for AFLP. AFLP has already been used to study the diversity of race 3 isolates of *R. solanacearum* (Wolf *et al.*, 1998). Poussier *et al.*, (1999) reported a simple, rapid method for discriminating *R. solanacearum* strain from *P. syzygii* and BDB strains. Its reliable determination of genetic relation between strains is usefulness for epidemiological and phylogenic purposes (Gillings and Fahy, 1994; Keim *et al.*, 1997; Fegan *et al.*, 1998; Tsuchiya and Horita, 1998).

Yan *et al.* (1999) compared estimates of population genetic parameters between AFLPs and RFLPs in populations of the yellow fever mosquito, to prove that the Hardy–Weinberg equilibrium is strictly followed in AFLP markers.

A highly resistant and primitive species *Solanum phureja* was employed to generate a mapping population to perform the BSA for screening of AFLP markers linked with the resistance to *Ralstonia solanacearum*. Another population which had genetic similarity to the mapping population was used for testing of the markers achieved and common AFLP marker strategy was used to identify the genomic position of the molecular markers in the linkage map. AFLP markers ATG/CTC 307.0, ATG/CTC 246.0, ATG/CTC 191.0 and AAC/CAC 79.0 were considered as markers that associated with the bacterial wilt resistance in potato and located on the relevant chromosome maps (Gang *et al.*, 2005).

AFLP was conducted on a set of 92 *Nicotiana tobacum* L. accessions from diverse types and breeding origins to identify markers associated with disease

resistances. Seven fragments were associated with three different resistances: two for the blue-mold (*Peronospora tabacina* Adam) resistance derived from *Nicotiana debneyi* Domin, two for the *Va* gene (Potato Virus Y susceptibility), and three for the black root rot resistance of *N. debneyi* origin. Some of these markers were converted into SCAR markers, and validated on recombinant inbred lines or doubled-haploid lines (Julio *et al.*, 2006).

Amplified fragment length polymorphism (AFLP) marker was potentially identified to be linked to the tomato spotted wilt virus resistant gene (Price *et al.*, 2007) and the gene was mapped on chromosome 12 flanked by the markers T1263 (45.0 cM). The rice gene *Xa21* conferring resistance to *X. oryzae pv. oryzae* (*Xoo*) of bacterial leaf blight and blast which was isolated using a map-based cloning strategy (Ronald, 1997). RAPD markers extended the *S. commersonii* population analyzed previously (Pianzzola *et al.*, 2005) by AFLP and SSR techniques to evaluate genetic variation among wild forms of *S. commersonii* collected from different locations in Uruguay (Siri *et al.*, 2009).

cDNA-AFLP is a powerful technique for investigating the expression pattern of chickpea genes under low-temperature stress (Dinari *et al.*, 2013). The quantitative real-time PCR (RT-PCR) validate the genes differentially expressed in interactions between tomato genotypes and *R. solanacearum* and identified using cDNA AFLP. Nazeem *et al.* (2011) reported comparison of transcript profiles generated from the tomato genotypes, viz., Anagha (resistant) and H24 (susceptible) at second and fifth day after inoculation by subjecting them to cDNA-AFLP analysis. A total of 763 transcript-derived fragments (TDFs) were analyzed from 21 primer sets and 58 TDFs were detected to be differentially expressed during pathogen challenge. The transcription factor Retrotransposons, Aspartate kinase/homoserine dehydrogenase, NBS-LRR and Ribonuclease H were identified which represented key factors governing detection and activation of defense mechanism in cultivated tomato genotypes with respect to bacterial wilt incidence.

2.8.2. Capillary electrophoresis

Recently high-throughput genotyping techniques are developed which allow marker aided screening of many genotypes and capillary electrophoresis is a typical example. The initial use of microsatellite markers was based on agarose gel assays, and even today construction of linkage maps and diversity analysis in plants are usually based on agarose gel assays. However, forensic genetic analysis requires higher resolution fragment separation and greater accuracy in allele sizing because diagnostic markers often possess alleles with as little as 2 bp size difference. Thus, polyacrylamide gel electrophoresis (PAGE) was recruited to achieve better fragment separation, and agarose gel electrophoresis was soon replaced by sophisticated and semi-automated PAGE platforms (Butler *et al.,* 2004). However, a major upward shift in the accuracy of microsatellite marker based forensic analysis took place with the introduction of capillary electrophoresis technology by various manufacturers, for example, Applied Biosystems (ABI310, ABI3100, ABI3700, ABI3730), Amersham Biosciences (MegaBASE500, 1000, 4000), Beckman Coulter (CEQ8800), and SpectruMedix Corporation (SCE2410, 9610, 19210) (Koumi *et al.,* 2004). Capillary electrophoresis heralded accurate and consistent allele sizing with minimal manual intervention, thereby reducing the sizing error, the technique was subsequently extended to animal and plant variety identification and high-density genetic mapping projects (Altet *et al.,* 2001; Tang *et al.,* 2004).

During capillary electrophoresis, the products of the PCR reaction enter the capillary as a result of electrokinetic injection. A high voltage charge applied to the sample forces the negatively charged fragments into the capillaries. The extension products are separated by size based on their total charge. Shortly before reaching the positive electrode, the fluorescently labeled DNA fragments, separated by size, move across the path of a laser beam. The laser beam causes the dyes attached to the fragments to fluoresce. The dye signals are separated by a diffraction system, and a CCD camera detects the fluorescence Applied Biosystems 3130 Genetic Analyzer belongs to the latest generation of 4-capillary

electrophoresis instruments for the low to medium throughput laboratories (Polanco *et al.,* 2005).

The single-base resolving capability of capillary electrophoresis permits all four fluorochrome products to be separated simultaneously compared to classical sequencing with PAGE, where each chain terminator has to be loaded in separate lanes. The result is a multicolored ladder where each color represents a different base. The order of the bases is then analyzed using secondary software that characterizes the sequence in terms of identity, and relatedness to prototypical sequences in a database (Tan and Yeung, 1998; Zhang *et al.,* 1999).

Polanco *et al.* (2005) have developed a method that allowed for isolation and cloning of specific AFLP markers obtained with a laser-induced fluorescence capillary electrophoresis system. This procedure has been tested on five *Arabidopsis thaliana* polymorphic AFLP markers, and the nucleotide sequences obtained from these cloned markers were identified.

2.9 Bulk Segregant Analysis (BSA)

BSA involves two different groups of plants according to either high or low expression of a particular trait and extracting DNA from these two bulks. Bulked DNAs will be prepared from equal volumes of standardized DNA of up to 10 each of resistant and susceptible F_2 plants. By grouping plants according to either high or low expression of a particular trait and extracting DNA from these two bulks, the process of genotyping the plants is reduced to only two DNA samples to be analysed instead of having to analyse DNA separately from each plant. It map specific region of the genome. BSA analyse these DNA samples which are identical for trait of interest but arbitrary for all other genes. This methodology was applied to identify molecular markers linked to a major fertility restorer gene *(Rf)* using the F_2 population of Niujiaojiao No.21 (rfrf)/Xiangtanwan (RfRf). Two random amplified polymorphic DNA (RAPD)

markers linked to this allele were detected with 520 decamer primers with arbitrary sequences. OP131400 was a tightly linked marker with a genetic distance of 0.37 cm. OW19800 is on the opposite side with a distance of 8.12 cm (Baoxi *et al.*, 2000).

The AFLP combined with BSA is at present considered to be the most powerful method in both searching for linked markers and for saturating the target region (Vos *et al.*, 1995; Ballvora *et al.*, 1995; Thomas *et al.*, 1995; Qi *et al.*, 1998; Xu *et al.*, 1999). The essence of the BSA is to create a bulk sample of DNA for analysis by pooling DNA from individuals with similar phenotypes. Michelmore *et al.* (1991) had used BSA to identify three random amplified polymorphic DNA markers in lettuce linked to gene for resistance to downy mildew.

Two variants of the BSA technique are possible depending on whether these plants are derived from a cross between two parental lines or well from a population of plants with diverse genetic backgrounds. In a modified QTL analysis, BSA was used to identify the genes determining the drought resistance of maize (Quarrie *et al.*, 1999). BSA with cDNAs of stress-induced genes used as probes hybridized to populations selected on the basis of differences in stress responses or resistance could be used as a simpler alternative to transformation (Quarrie *et al.*, 1996). BSA in combination with the AFLP markers was used to identify molecular markers associated with the bacterial wilt resistance genes in tomato and then converted this linked marker to a SCAR marker (Miao *et al.*, 2009).

2. 10 Use of AFLP markers to tag resistance genes using BSA on NILs

A bulk segregant analysis approach (Michelmore *et al.*, 1991) was followed in which the two extreme bulk samples provided a crude simulation of NILs. A process of elimination of unlinked markers minimized the number of

samples to be tested. This approach was highly efficient for identifying associated markers, especially when dealing with monogenic actions.

AFLP assay is efficient for analysing multiple loci of an individual which translated to homozygous or heterozygous genotypes at each locus, and allelic structure derived is utilized for comparison between individuals (Dalirsefata *et al.*, 2009). AFLP followed to tag major gene by construction of near isogenic line in parental and progeny polymorphic loci. Zhang *et al.* (2013), screened AFLP markers that were tightly associated with drought stress gene in both male sterile and fertile NILs of *Salvia. miltiorrhiza* and to examine the fertility change for fertile plants under severe drought stress conditions. They identified two AFLP markers (E9/M3246 and E2/M5357) in treated fertile and treated sterile NILs of *S. miltiorrhiza*, respectively, both tightly linked to the drought stress trait/gene.

Prins *et al.* (2001) and Blaszczyk *et al.* (2005) reported leaf rust resistance gene in wheat by tagging of Lr19 through AFLP and STS mapping of DNA marker technique. Li, (2010) identified AFLP fragments linked to soybean mosaic virus resistance gene (*Rsv*) in *Glycine soja* by BSA followed by conversion of AFLP to a SCAR marker for rapid selection. Miao *et al.* (2009) analyzed the mode of inheritance of bacterial wilt resistance in a segregating population derived from a pair cross between resistant and susceptible tomato cultivars by AFLP. Dhillon and Dhaliwal (2011) reported molecular markers linked with *Lr9*, *Lr19* and KLM4-3B genes, which provide resistance against most of the leaf rust pathotypes.

AFLP analysis carried out in NILs of Thatcher carrying different genes conferring resistance against wheat leaf rust and TcLr45 x Thatcher F_2 progenies were used to develop markers for *Lr45* gene (Na *et al.*, 2006). Using AFLP, bacterial wilt resistance in tomato was evaluated in F_3-progenies derived from two crosses between near-isogenic lines Caraïbo x Carmido and CRA 66 x Cranita, differing for small and large introgressions from *Lycopersicum*

peruvianum that carry the *Mi* gene which offer resistance to bacterial wilt.. The *Mi* gene and a major QTL for resistance to bacterial wilt were mapped to tomato chromosome 6 in tomato (Deberdta *et al.,* 1999).

2.9.1 Mapping QTLs using AFLP markers

QTL analysis allow direct study of genes and gene clusters involved in bacterial wilt resistance and an understanding of the expression of these genes and their interaction with environment and phenotype. Naidoo *et al.* (2003) have used AFLP to map the QTLs linked to common bacterial blight (*X. axonopodis pv. phaseoli*) resistance, and Kolkman and Kelly (2003) to identify QTLconferring resistance to white mold (*Sclerotinia sclerotiorum*). QTL markers have also been developed for ashy stem blight (*Macrophomina phaseolina*), bean golden mosaic virus (BGMV), and web blight (*Rhizoctonia solani*) (Kelly and Miklas, 1999; Kelly *et al.*, 2003).

AFLP coupled with BSA allowed the detection of QTLs governing rice grain chalkiness (Liu *et al.*, 2011). Thorogood *et al.* (2001) found QTL linked to crown rust resistance in rye grass. The susceptible variety 'Aurora' and resistance variety 'Perma' were screened by 45 RFLP and 27 AFLP polymorphism, for analyzing the association between the marker and crown rust resistance gene by drawing linkage map of 7 linkage group. Two QTL for crown rust resistance were identified at an interval of 5cM linkage group. Zhou *et al.* (2002) determined AFLP linkage group containing a major quantitative trait locus (QTL) for scab resistance in a mapping population of 133 recombinant inbred lines (RILs) derived from 'Ning7840' × 'Clark' and showed that the major QTL was located in a chromosome region about 8 cM in length around Xgwm533 and Xbarc147.

2.15. Marker Assisted Selection (MAS)

Breeders want to incorporate agronomically interesting traits in their breeding material. MAS are a technique that can facilitate this process by the selection of genes that control traits of interest. Development of disease resistant varieties is one of the most economical methods of control of diseases like bacterial wilt. It is often difficult to pyramid more than one important genes through conventional means, particularly where the resistance in question should be effective against all the prevalent pathotypes (Lowe *et al.*, 2011). However, recent advances in molecular biology had made it possible to pyramid several genes in single line using MAS. In MAS for disease resistance, the level of disease resistance is not quantified but a marker allele that is linked with disease resistance is used. For marker assisted selection, it is essential that markers are available that are diagnostic for the trait of interest (Moloney *et al.*, 2010). In MAS, the markers to be used should be close to gene of interest (<5 cM) in order to ensure that only minor fraction of the selected individuals will be recombinants. Generally, not only a single marker but rather two markers are used in order to reduce the chances of an error due to homologous. Before applying MAS in a breeding program, it must be validated that the markers used are polymorphic in the populations studied (Kumar *et al.*, 2009). Specific marker developed on the resistance gene itself has the highest chance of being diagnostic (Tomczak *et al.*, 2011). Throughout the world, PCR based markers are increasingly used to detect the introgressed genes in the segregating breeding populations due to the enormous amount of time that could be saved, freedom from environmental influences and accuracy. The highly successful initial attempts such as MAS for *Sw-5* gene of tomato spotted wilt virus resistance (Folkertsman *et al.*, 1999; Garland *et al.*, 2005; Shi *et al.*, 2011) have demonstrated and boosted its potential.

Materials and Methods

3. MATERIALS AND METHODS

The research study on "Identification of AFLP marker linked with bacterial wilt resistance in chilli *(Capsicum annuum* L.)" was conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period of 2011-2013. The materials used and methodologies adapted in the process are discussed in this chapter.

3.1. MATERIALS

3.1.1. Plant Materials

Seeds of the three chilli genotypes *viz.*, Ujwala, Anugraha and Pusa Jwala, having different responses to bacterial wilt disease (caused by *Ralstonia solanacearum*) were collected from different institutions (Table 1 and Plate 1). The F_2 progenies of the cross Anugraha (♀) x Pusa Jwala (♂) were used for screening against bacterial wilt and for genotyping by AFLP marker.

3.1.1.1. Rationale in selection of genotypes in the study

The superiority of F_2 generations originating from Near Isogenic Lines in screening the polymorphic bands against a specific character is well established. The bacterial wilt resistant chilli variety Anugraha was released from Kerala Agricultural University through back cross breeding from the cross of bacterial wilt resistant Ujwala (donor parent) and high yielding but bacterial wilt susceptible Pusa Jwala (recurrent parent). Thus, Pusa Jwala and Anugraha are near isogenic lines (NILs) differing in response to bacterial wilt. Since the Anugraha is holding the same gene for bacterial wilt resistance as in Ujwala, it was worth including Ujwala in the screening programme for further confirming that the band we have chosen for the character is correct.

Table 1. Sources of chilli genotypes

Sl. No.	Genotypes	Response to bacterial wilt disease	Source
1	Ujwala	Resistant	Krishi Vigyan Kendra, Thrissur, Kerala Agriculture University
2	Anugraha	Resistant	Department of Olericulture, College of Horticulture, Kerala Agriculture University, Thrissur
3	Pusa Jwala	Susceptible	National Seeds Corporation, New Delhi

a.Resistant variety Ujwala

b.Resistant parent Anugraha

c. Susceptible parent Pusa Jwala

d. F_2 progeny

Plate 1.Genotypes selected for the study

3.1.2. Laboratory chemicals and glassware

The chemicals used in this study were of good quality (AR grade) procured from Merck India Ltd., SRL, HIMEDIA and SISCO research Laboratories. The Taq DNA polymerase, Taq buffer and molecular weight marker (λDNA/*HindIII*+*EcoRI* double digest) were supplied by Banglore Genei. RNase A from sigma, USA was used. The plastic wares used for the study were purchased from Tarsons India Ltd., and Axygen, USA.

3.1.3. Equipment and machinery

The equipment available at the centre for Plant Biotechnology and Molecular Biology and the Bioinformatics Centre, College of Horticulture was used for the present study. Centrifugation was done in KUBOTA 6500 centrifuge. Dai Ki-S1010 (Dai Ki Scientific Co.) was used for the incubation of culture. The PCR was done in Eppendorf Master Cycler (Eppendorf, USA) and agarose gel electrophoresis done in horizontal gel electrophoresis system (BIO-RAD). Gel DOC-It ^{TM} Imaging system UVP (USA) was used for imaging the gel. The list of laboratory equipment used for the study is provided in Annexure I.

3.2. METHODS

3.2.1. Development of segregating population

3.2.1.1. Nursery raising and transplanting for confirming the resistance in parent plants

It is possible that even in a highly resistant population, a minor percentage of plants deviate from the expected resistance level. Though the possibility is remote, if the resistant and susceptible parents to be used in the study are chosen without sufficient field screening, it may end up in wrong parents. To avoid such a situation, resistant parent Anugraha and susceptible parent Pusa Jwala was initially field screened. The seeds of both the varieties were sown in pots on 4

Hybridization and development of F_1 plants

Plate 2. View of experimental plot

February 2012 and the seedlings were transplanted to open field on 23 March 2012.

3.2.1.2. Design of plots for screening the parents

For identifying highly resistant and susceptible parents screening was done on transplanted plants where the field was laid behind Cashew nursery. The area designed for resistant and susceptible plants was 7 x 3m with spacing 45 x 60cm respectively and in each plot of resistanat and susceptible 60 plants were maintained. Standard package of practices were followed in crop raising (KAU, 2011) and on 25 days after transplanting, plants were subjected to artificial inoculation to confirm the resistance and susceptibility for bacterial wilt. The methodology followed in artificial inoculation of bacterium for screening for resistance is detailed under 3.2.2.1.

3.2.1.3. Standardization of artificial inoculation technique

A pot culture study was carried out using the susceptible variety Pusa Jwala, to standardize inoculation technique in chilli. The seeds of Pusa Jwala and Anugraha were sown in sterilized pots containing 1:1:1 mixture of sand, soil and FYM. Sterilization of the medium was carried out with 40 per cent formaldehyde solution. The fresh bacterial ooze from wilted plants was used for inoculation. The bacterial ooze was collected in 100 ml of sterile distilled water and the optical density (OD) of the suspension was adjusted to 0.3 at 600 nm containing 10^8 cfu/ml and screening was done during the month of May 2012 in open condition. One month after sowing, when the seedlings are at 10-15 cm height, they were transplanted to sick pots and inoculated with fresh bacterial ooze. The following three methods (Plate 3b) of inoculation were tested for identifying the most suitable methodology.

a. Transplanted F$_2$ progenies

b. Artificial inoculation technique

Plate 3. Screening of F$_2$ plants

i) Stem-puncturing:

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. The treatment was given after twenty five days transplanting.

ii) Soil drenching with wounding:

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

iii) Pin pricking

Pin or needle was dip into bacterial suspension and then pricked into the leaf through making small holes the treatment was given 25 days after transplanting seedlings. This is most successful inoculation method. Plants were observed for 15 days after incidence of bacterial wilt. The wilted plants were subjected to ooze test to confirm the association of pathogen with the wilt (Plate 5b). The severity of wilt incidence was scored according to the resistance/susceptible based on scoring system by Mew and Ho (1976) and details are given below

Disease Rating	Per cent survival
R (Resistance)	80 or above
MR (Moderately resistance)	60-80
MS (Moderately susceptible)	40-60
S (susceptible)	Less than 40

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

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

Thus the susceptible plants from the resistant parental population and resistant plants from susceptible parental population were identified and removed and only the plants showing the true disease reaction were retained.

3.2.1.4. Hybridization and development of F_1 plants

The hybridization of the selected parents was done under open field conditions. The bacterial wilt resistance variety Anugraha was crossed with the pollen from susceptible variety Pusa Jwala for development of F_1 hybrids (Plate 4c.). The reciprocal cross was not possible because the susceptible parent when used as female parent had resulted in complete plant wilting before fruit maturity. From the susceptible parents, pollen was collected at the onset of wilting symptoms and directly used in hybridization. Female flowers on resistant plants which were ready to open in the next day morning were emasculated in the evening at 5-6 pm. Artificial pollination was done at 7 am on the day of anthesis (Plate 2.). After pollination, the pollinated flowers were covered with butter paper bags and tagged properly. Ripe fruits from such crosses were carefully harvested, shade dried and seeds were extracted manually and maintained under refrigerated condition till the sowing for raising the F_1 crop.

3.2.2. Field raising and screening the F_1 plants

F_1 seeds were sown in sterilized pots containing 1:1:1 mixture of sand, soil and FYM. Sterilization of the medium was carried out with 40 per cent

formaldehyde solution under green house condition on 9 August 2012. Among all 110 raised F_1, 10 plants were kept into the pots for developing F_2. But in order to verify the gene action, an open field was raised for the remaining F_1 crop in bacterial wilt sick plot behind the College of Horticulture and inoculated by above (3.2.1.3.) three methods of artificial inoculation.

3.2.2.1. Design and layout of F_1 field

To raise the F_1 crop, the crossed seeds from the parental field were sown in pots and seedlings were transplanted on 24 September 2012. The area selected for raising 100 F_1 was 3 x 5 m in three plots with spacing 45 x 60cm. The artificial inoculation was practised as detailed above and the ratio of susceptible and resistant plants was recorded. Peter *et al.,* (1992) reported that the resistance to bacterial wilt is offered by homozygous recessive condition *rr*. Hence we have to expect complete susceptibility in F_1 generation (*Rr*) and resistance if found will be due to the heterogenic allelic condition in the selected parents. Under such situation, it was decided that the susceptible F_1 has to be selfed to develop the F_2 generation. If the susceptible F_1 is used for selfing under field conditions, crop will not be completed and it will not be feasible to harvest the F_2 seeds. So 10 F_1 plants were simultaneously raised in pots under greenhouse condition, flowers were selfed and seeds were harvested. After seed harvest, these plants were taken out of the greenhouse and artificially inoculated with bacterial culture and resistance was checked. The plants showing the susceptibility were identified and the seeds from those plants were stored for raising the F_2 segregating population.

3.2.3. Raising the F_2 progeny

Two hundred F_2 (Plate 3a) plants germinated from the seeds of a single F_1 hybrid were used as mapping population. On 18 February 2013 approximately 300 F_2 seeds were sown in pots inside greenhouse (Plate 4d). The seedlings were transplanted to the disease screening plot at 15 April 2013 (Plate 3a). Total area

Table 2. Layout of field

Sl. No.	Genotype/ Generation	Area (m^2)	Spacing (cm)	No. of plants	Plots
Parental Generation					
1	Anugraha	21 (7 x 3)	45 x 60	60	1
2	Pusa Jwala	21 (7 x 3)	45 x 60	60	1
F$_1$ Generation					
3	F$_1$	15 (5 x 3)	45 x 60	100	3
F$_2$ Generation					
4	F$_2$	45 (9 x 5)	60 x 60	200	2

designed for 200 plants was 9 x 5m sized two plots. Total 30 rows were taken with spacing 60 x 60 cm.

3.2.3.1. Phenotyping of genotype for bacterial wilt resistance

The seedlings were subjected to artificial inoculation with bacterial inoculum by pin pricking and stem puncturing methods on 10 May 2013 at 25 days after transplanting. The first 10 wilted plants were considered as most susceptible. At the onset of the wilting symptoms, tender leaves from these plants were collected and DNA was extracted. The plants those withstood the intensive bacterial inoculation even towards the end of the crop were considered as most resistant and DNA was extracted from 9 resistant plants. From among the most susceptible plants, first 10 plants were identified and DNA was extracted from these plants.

Chi-square test was employed to test the segregation ratio for disease incidence. This statistic was computed using the formula given below

$$\text{Chi square test } (\square^2) = \frac{[\text{Observed mean (O)} - \text{Expected mean (E)}]}{\text{Expected mean (E)}}$$

3.3. Molecular characterization of the mapping population by AFLP Assay

Molecular characterization of selected bacterial wilt resistant and susceptible NILs, their F_2 resistant and susceptible progenies and the donor parent for bacterial wilt resistance gene was done using AFLP (Plate 6) markers. The capability of this marker to generate large number of bands in comparison with the other commonly used markers such as RAPD, SSR or ISSR was the rationale in selecting this marker system for this study. Thus the probability of detecting the polymorphism will be much higher through this strategy.

3.3.1. Genomic DNA extraction

Tender emerging leaves (first to third from the tip) were collected early in the morning on ice from individual plants. The collected leaves were quickly covered in aluminium foils and transported to the laboratory in ice flask. The surface was cleaned by washing with sterile water and wiping with 70 percent ethanol and stored at -80°C till being used. CTAB method developed by Rogers and Bendich (1994) was used for the extraction of genomic DNA.

3.3.1.1. Reagents

a. CTAB buffer (2X)

b. 10% CTAB solution

c. TE buffer

d. Chloroform: isoamyl alcohol (24:1) v/v

e. Chilled isopropanol

f. Ethanol 70%

The details of preparation of reagents are provided in Annexure II

3.3.1.2. Procedure

One gram of cleaned leaf tissues was taken in autoclaved ice-cold mortar and pestle (in order to prevent brewing due to phenol oxidase activity). Four ml of extraction buffer (2x) and 50μl of β-Mercaptoethanol were added with 1 pinch of PVP. Ground the leaf tissue into fine powder in liquid nitrogen and transferred the homogenized sample into an autoclaved 50ml centrifuge tube and 3ml of pre-warmed extraction buffer. Mixture was mixed well and incubated at 65°C for 30 minutes with occasional mixing by gentle inversions. Equal volume (7ml) of chloroform-isoamyl alcohol (24:1) added and mixed by gentle inversions to emulsify and spun at 10,000 rpm in cold centrifuge (Kubota 6500) for 15 minutes at 4°C. After centrifugation, the contents got separated into three distinct phases. Aqueous top layer containing DNA with small quantity of RNA (removed by RNase treatment as given in 3.3.3.), middle layer having protein, fine particles

and lower layer containing chloroform, pigments and cell debris. So, transferred the top aqueous layer to a sterile centrifuge tube and 1/10th volume of 10 percent CTAB solution and equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion centrifuged at 10,000 rpm for 15 minutes at 4°C.Transferred the aqueous phase into a clean centrifuge tube and added 0.6 volume (3 ml) of chilled isopropanol and mixed by gentle inversions till the DNA precipitated. These tubes were kept at -20°C for half an hour for complete precipitation. After the expiry of time, centrifuged at 10,000 rpm for 15 minutes at 4°C and the supernatant was gently poured off. The DNA pellet was washed with 70% ethanol and spun tubes for 5 min at 10,000 rpm and ethanol was decanted. The pellet was air dried, dissolved in 50µl sterile distilled water and stored at -20°C

3.3.2. Assessing the quality of DNA by electrophoresis

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

3.3.2.1. Reagents and Equipments

a. Agarose 0.8 percent- Promega
b. 50X TAE buffer (P^H 8.0)
c. Tracking/loading dye (6X) – Banglore Genei
d. Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml)- SRL
e. Electrophoresis unit- BioRad power PAC 1000, gel casting tray, comb
f. UV translluminator- (Herolab [R])
g. Gel documentation and analysis system- BioRad Gel DOC-It [TM] Imaging system

Composition of reagents is provided in Annexure III.

3.3.2.2. Procedure

The gel tray was prepared by sealing the ends with tape. Comb was placed in gel tray about 1 inch from one end of the tray and positioned the comb vertically such that the teeth are about 1 to 2 mm above the surface of the tray. (0.8%) Agarose (0.8 g in 100ml sterile distilled water) was prepared in a glass beaker or conical flask with 100 ml 1X TAE buffer. Microwaved for 45 to 60 seconds until agarose was dissolved and solution was clear. Solution was allowed to cool at 42 to 45 ^{0}C, ethidium bromide was added at this point a concentration of 0.5µg/ml and poured to gel casting tray to a depth of about 5 mm. The gel was allowed to solidify for about 30 to 45 minutes at room temperature. To run, the comb and the tape used for sealing were gently removed and the tray was placed in electrophoresis chamber. The gel was covered with electrophoresis TAE buffer till the wells are submerged. Samples for electrophoresis were prepared by adding 1 µl of 6x gel loading dye for every 5µl of DNA solution and mixing them well. Loaded 6µl DNA per well. A suitable molecular weight marker (λDNA *Eco*RI/ *Hind*III double digest- Banglore Genei) was also loaded in one lane. Electrophoresis was carried out at 70 volts until dye has migrated two-third the length of the gel. Intact DNA appeared as orange fluorescent bands. The degraded ones appeared as a smear because of the presence of a large number of bands, which differed only in one or two bases.

3.3.3. Purification of DNA

The DNA which had RNA as contaminant (as observed from the electrophoresis) was purified by RNase treatment and precipitation was followed (Sambrook *et al.,* 1989).

3.3.3.1. Reagents

I.   Phenol: chloroform mixture (24:1, v/v)

II.   Chilled isopropanol

III.   70 per cent ethanol

IV.   50 X TAE buffer

V.   Chloroform: Isoamyl alcohol (24:1, v/v)

VI.   1% RNAse

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

3.3.3.2. Procedure

For 50 µl DNA sample, 1 µl of 1 per cent RNase solution was added and incubated at 37^0C in dry bath for 40 minutes and total volume was made up to 250 µl with distilled water. Equal volume of chloroform: isoamyl alcohol (24: 1) mixture was added and mixed gently and centrifuged at 15000 rpm for 15 minutes at 4^0C. The aqueous phase was transferred into a fresh micro centrifuge tube and equal volume of chloroform: isoamyl alcohol (24: 1) was added and centrifuged at 12,000 rpm for 15 minutes at 4^0C. The aqueous phase was transferred into a clean centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by 2-3 gentle inversion till the DNA precipitated. For complete precipitation kept it at -20°C for half an hour. The mixture was incubated at 20^0C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4^0C. DNA pellet was washed with 70 per cent ethanol and centrifuge at 10,000 rpm for 10 minutes at 4^0C. The pellet was air dried and dissolved in 50 µl sterile distilled water. The samples were loaded on 0.8 per cent agarose gel at constant voltage of 70V to test the quality and to find whether there is any shearing during RNAse treatment.

3.3.4. Gel Documentation

The gel containing electrophoresed DNA was viewed under UV transilluminator for presence of DNA. The DNA fluoresces under UV light due

to ethidium bromide dye. The image was documented on gel documentation system (BioRad Gel DOC-It ^{TM} Imaging system imaging system). The gel profile was examined for intactness, clarity of DNA and contamination with RNA and protein.

3.3.5. Assessing the quality and quantity of DNA by Spectrophotometer (NanoDrop ND-1000) method

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

1OD at 260 nm = 50 μg double stranded DNA/ml of the sample.

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

3.3.5.1. Procedure followed for quantity check using Nanodrop Spectrophotometer

Nanodrop spectrophotometer was connected to the system and the operating software ND-100 was opened, option Nucleic acid was selected with the sampling arm open, 1μl distilled water was pipetted onto the lower measurement pedestal. Sampling arm was closed and spectral measurement was initiated using the operating software on the PC. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement was made. The reading was set to zero with sample blank pipetted out 1μl sample onto measurement pedestal and measure option was selected. When the measurement was completed, the sampling arm was opened and the sample was wiped from both the upper and lower pedestals using a soft laboratory tissue paper.

3.3.6. Amplified Fragment Length Polymorphism (AFLP)

AFLP is highly sensitive and reproducible method for detecting polymorphism throughout the genome. It is based on PCR amplification of genomic restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of a few nucleotide bases (Vos *et al.*, 1995). Standard AFLP kit (catalog no. 10544-013 and 10483-014) provided by *Invitrogen* was initially attempted. The pre-amplification and selective amplification were done and the products were resolved using polyacrylamide gel electrophoresis (PAGE). The PAGE gel was developed by using co-polymerization of acryl amide and a cross linker, N'N'-methylene bisacrylamide. The ratio of acryl amide to bisacrylamide (19:1 is commonly used) and total concentration of acryl amide in gel determine the pore size of the matrix and its sieving effect on the DNA molecule as they pass through it. To keep the DNA denatured, the sequencing gel was prepared with a high concentration of urea (8M). Formamide dye was added to the gel to increase its denaturing capacity. Electrophoresis was done at constant high voltage (1200V) and the power was adjusted to maintain the gel temperature at 55 to 60°C. Electrophoresis took about 2hrs time and observed for amplicons. Though this methodology was repeated for more than 25 times by altering the pre-amplification and selective amplification conditions and the primer combinations, the Invitrogen AFLP kit has failed to yield results. Under these conditions, it was decided to adopt the AFLP research kit provided by Chromous Biotech (Research kit manual) Bangalore. Unlike the costly Invitrogen kit which was meant for 6000 samples, the Chromus kit was cost effective and aimed for only 10 samples.

The steps involved in the AFLP analysis were

1. Restriction endonuclease digestion of the genomic DNA
2. Ligation of adapters
3. Pre-Amplification of restriction fragments
4. Selective amplification with fluorescent labeled primers

5. Gel analysis of the amplified fragments

6. Detection of bands of genetic analyzer

Different combination of *Eco*RI and *Mse*I primer pairs were used for different AFLP reactions. This method allowed specific co-amplification of 100-200 restricted fragments in each reaction and detection on capillary electrophoresis. The resulted banding pattern was documented and analyzed for polymorphisms using analytical software Data collection 2.2 and GeneScan 2.2.

3.3.6.1. Protocol for AFLP assay

The AFLP analysis was carried out using AFLP analysis kit (Chromous Biotech) according to instructions provided. Composition of different reagents used in AFLP analysis is given in Annexure IV. The procedure followed involved the following steps

A) Restriction Digestion of Genomic DNA

The components were added to a 1.5ml microfuge tube and the digestion reaction was set up.

Components	Sample
10X Assay buffer	5 μL
Sample genomic DNA (250ng)	1.5 μL
*Eco*RI (10U/μL)	0.5 μL
*Mse*I (10U/μL)	0.5 μL
Sterile grade I water	42.5 μL
Total volume	50 μL

The contents were mixed gently and a brief centrifugation was given. The mixture was incubated for 2hrs at 37°C. The mixture was again incubated at 70°C

for 15 min to inactivate the restriction endonuclease. Then the tubes were placed on ice and the contents were collected by brief centrifugation. Purified the restricted DNA sample by following DNA precipitation protocol

1/10th volume of 3M sodium acetate (pH 5.2) and equal volume of isopropanol were added to the digested sample and left at room temperature for 10 minutes and centrifuged at 10,000 rpm for 15-20 minutes at 4°C. Supernatant was gently poured off. DNA pellet was washed with 70% ethanol followed by 100% ethanol and spun for 5 min at 10,000rpm and decanted the ethanol. The pellet was air dried for 5 minutes and dissolved in 50µL sterilized water and stored at -20°C.

B) Ligation of Adapter

The following components were added to purify digested DNA in a 1.2ml microfuge tube.

Components	Sample
2.0 X quick ligase assay buffer	30 µL
Digested DNA (purified)	20 µL
*Eco*RI adapter	0.5 µL
*Mse*I adapter	5.0 µL
Quick ligase	1.0 µL
Water	3.5 µL
Total volume	60 µL

The sequence of the adapter *EcoRI* and *MseI* used are listed in Table 3.

Table 3. List of adapter sequences used in AFLP adapter ligation

Sl No.	Adapters	Sequences
1	*EcoRI*	
	Eco A1:	5'- CTCGTAGACTGCGTACC-3'
	Eco A2:	5'- AATTGGTACGCAGTCTAC-3'
2	*MseI*	
	Mse A1:	5'- GACGATGAGTCCTGA G-3'
	Mse A2:	5'- TACTCAGGACTC AT-3'

The contents were mixed gently and centrifuged briefly. These tubes were incubated at 20±2°C for 2 hrs. Performed 1:5 dilution of the ligation mixture by mixing 10μL of ligation mixture in to 40μL of autoclaved distilled water and mixed well. The unused portion of the reaction mixture was stored at -20°C.

C) Pre-amplification Reactions

PCR amplification was carried out in two consecutive steps. In the first reaction, called pre-amplification, the ligated product was amplified with preamp primer containing one selective nucleotide (N+1). The reaction was set up as given below. The following components were added to a 0.2 ml thin walled microfuge tube.

Components	Sample
Diluted template DNA (1:5 ligated product)	5.0 μL
Pre-amp primer mix	2.0 μL
10X chromo Taq assay buffer	2.5 μL
dNTPs (2.5 mM each)	2.0 μL
ChromoTaq DNA polymerase (3U/ul)	0.5 μL
Water	13.0 μL
Total reaction volume	25 μL

The content in the tubes were mixed gently and briefly centrifuged to collect the reaction. The tubes were placed in a thermal cycler and PCR was performed for 25 cycles at

94°C for 5 min for initial denaturation

94°C for 30 sec for denaturation

55°C for 30 sec for annealing

72°C for 2 min for primer extension

72°C for 5 min for final extension and

4°C for soaking

Following amplification, 1:50 dilution of the PCR product was done as follows: 1 µL of the amplified product was transferred to a fresh 0.2mL micro centrifuge tube containing 49 µL of autoclaved distilled water. Both the diluted as well as undiluted reaction products were stored at -20°C.

D) Selective AFLP Amplification

The 1:50 dilution of the amplified product was used as template for the selective amplification reaction using *MseI* and *EcoRI* primer containing 3 selective nucleotides (*Eco*ACT/*Mse*CAC). The sequence of the *EcoRI* and *MseI* used are listed in Table 4.

Table 4. List of selective primer used in AFLP

Sl No.	Selective primers	Sequences
1	EcoRIFAM	5'- CTCGTAGACTGCGTACCAATTCACT-3'
2	MseFAM	5'- GACGATGAGTCCTGAGTAACAC-3'

The following components were added to a 0.2ml microfuge tube and mixed it.

Component	Sample
Diluted template DNA (1:50 pre-selective amplified product)	5.0 μL
Selective primer mix (FAM labeled)	2.0 μL
10 X ChromoTaq assay buffer	2.5 μL
dNTPs 2.5 mM each	2.0 μL
ChromoTaq DNA polymerase (3U/μL)	0.5 μL
Water	13.0 μL
Total reaction volume	25 μL

The tubes (25μl reaction volume) were placed in a thermal cycler for selective amplification 35 cycles with following conditions

94°C for 5 min for initial denaturation

94°C for 30 sec for denaturation

55°C for 30 sec for annealing

72°C for 2 min primer extension and

72°C for 5 min for final extension

After completion of amplification, the product was soaked at 4°C. The product was then stored at -20°C for further use.

3.3.6.2. Analysis of selective- AFLP PCR product

The PCR products of selective amplifications were separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer and detected by mixing with internal size standard (LIZ 500 or ROX500) and Hi-di foramide with FAM labeled primers which gave blue color in genescan analysis. An internal size marker, Genescan Rox-500 (35-500bp: Applied Biosystems), labeled with carboxy-X-rhodamine dye, was added, that allowed the co-loading of different

labeled reactions. An internal size marker, the GeneScan™ 500 LIZ® Size Standard was a fifth dye-labeled size standard for the reproducible sizing of fragment analysis data. This size was used as standard for fragments between 35 and 500 bp. The standard contained 16 LIZ® dye-labeled, single-stranded DNA fragments.

3.2.3.7. Bulked Segregant Analysis (BSA) of chilli for bacterial wilt resistance

Bulk segregant analysis was carried out with DNA from resistant parent (Anugraha), susceptible parent (Pusa Jwala), resistant bulk (resistant F_2s), susceptible bulk (susceptible F_2s) and resistance donor Ujwala. Susceptible and resistant bulks consisted of DNAs from 9 plants. Selected primer combination as detailed in Table 3 was used for bulked segregant analysis.

PCR was carried out as per the procedure mentioned in section 3.3.6.1. The PCR amplified products of five groups of genomic DNA were loaded on to ABI genetic analyzer with capillary electrophoresis, by mixing with internal size standard (LIZ 500 or ROX500) and Hi-di foramide. The computer documented AFLP raw data for selected primer combination was carefully examined for polymorphism among susceptible parent, resistant parent, resistant bulk, susceptible bulk and resistance donor. The bands which showed polymorphism in resistant and susceptible genotype were identified.

3.2.3.8. Analysis of amplification profiles

Data regarding selectively amplified DNA fragments were collected by a computer connected to the ABI Prism 310 that was running Data Collection 2.1 software (Applied Biosystems) and were analyzed with GeneScan 2.2 software (Applied Biosystems), which sized and quantified the detected fragments. GeneScan software was also used to compare the electropherograms from the analyzed plants in order to detect the polymorphic AFLP markers. The output was

carefully examined to identify the polymorphic bands those are cosegregating with the character bacterial wilt resistance. Raw data bands base pairs were collected with GeneScan 2.2 software and scoring was done. The resulting data were analysed using the software package NTSYS pc version 2.02i (Rohlf, 1986).

Results

4. RESULTS

The results of the study entitled "Identification of AFLP marker linked with bacterial wilt resistance in chilli (*Capsicum annuum* L.)" undertaken during the period from 2011-2013 at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara are presented in this chapter.

4.1. Development of mapping population

For identifying the polymorphic bands for a specific character using marker systems, Bulk Segregant Analysis (BSA) is an accepted strategy (Michelmore *et al.*, 1991). BSA involves the development of hybrid population between the parents which are divergent for the charcter of interest, forwarding the hybrids to F_2 segregating population by selfing, screening of this segregating population and bulking the DNA of the F_2 plants which have the same character expression and using the DNAs from parental and bulked F_2s in marker analysis to determine the polymorphic bands. Near Isogenic Lines (NILs) are the accessions those are genetically similar except for a character under study. Pusa Jwala and Anugraha are NILs differing in response to bacterial wilt only. Hence in this study to identify the markers linked to the character bacterial wilt resistance, these two varieties were selected for mapping population development.

4.1.1. Standardization of artificial inoculation technique

Artificial inoculation technique was standardized in field cultured plants of susceptible variety Pusa Jwala and resistant variety Anugraha. Artificial inoculation was done 30 days after transplanting with fresh bacterial ooze collected from first susceptible plant. Artificial inoculation was done by 3 methods 1) leaf cutting 2) pin pricking (Plate 3b) and 3) soil drenching with stem

a.Pusa jwala (susceptible)

b.Anugraha (resistant)

c.Ujwala (resistant donor)

Plate 4. Genotypes and generation used for molecular charecterization of chilli genotypes

wounding at collar region. The susceptible genotypes showed wilting within 5 days in leaf cutting and pin pricking methods whereas in soil drenching with wounding it was 20 days. The wilted plants were subjected to ooze test (Plate 5) and *Ralstonia solanacearum* was confirmed to be the causal organism.

Per cent wilt incidence was recorded in above three methods (Table 5.). In both the methods, the susceptible parent has shown complete wilting. So based on quick response of plants for disease incidence, leaf cutting and pin pricking method was recommended to screen the population.

4.1.2. Screening the parental lines in open field

For confirming the resistance in the resistant parent Anugraha and susceptibility in susceptible parent Pusa Jwala and to choose the right plants for advancing the mapping populations, 60 plants each of Anugraha and Pusa Jwala were initially screened in the open field. The resistant and susceptible plants were artificially inoculated with the bacteria *Ralstonia solanacearum* and the responses were recorded. Among the resistant parental population, 6.66 per cent of plants were found to be susceptible for bacterial wilt. Similarly, in the susceptible population, 93.33 per cent wilt incidence was observed (Table 6.).

The most resistant plant from the population of Anugraha (Plate 6b) was crossed with the pollen from the most susceptible plant from the population of Pusa Jwala (Plate 6a) and the seeds for F_1 generation were harvested.

4.2. Genetic analysis for bacterial wilt resistance

4.2.1. Screening the F_1 generation for bacterial wilt resistance

The seeds for the F_1 generation were harvested from the selected and crossed Anugraha plant. These seeds were sown in pots and transplanted in open field and subsequently screened with artificial inoculation. It was observed that

approximately 50 per cent of the plants (58) have wilted giving nearly 1:1 ratio. This ratio has led to the proposal of two probable gene actions. First being the homozygous recessive gene action, provided the susceptible parent selected in the cross was heterozygous and the second being the dominant single gene action for resistance provided the resistant parent selected in the crossing was heterozygous.

As per the first proposal, if we are going for selfing the resistant rr plants, the F_2 progeny will not be segregating and necessarily, the susceptible Rr has to be used for advancing the generation. Since the susceptible plant is raised in open field for advancing the generation, there is chance for losing the plants before the maturity of the seeds. So pots and soil were sterilized and seeds were sown and plants were raised in greenhouse. Mature selfed seeds were harvested and maintained separately for each plant. After harvest of one ripe fruit, the plants were inoculated with bacteria and seeds from first wilted plant were advanced as F_2 population.

Table 5. Standardisation of artificial inoculation technique using different genotypes

Sl. No.	Methods	Name of plants	Days taken for initiation of wilting	% of wilting
1	Leaf cutting and pin pricking	Anugraha	0	0
		Pusa Jwala	5	100
		F_1	5	90
2	Soil drenching with stem wounding	Anugraha	0	0
		Pusa Jwala	20	100
		F_1	20	75

4.2.2. Screening the F_2 plants in open

F_2 seedlings were raised in pots (Plate 6d.) and 200 of them were transplanted to open field (Plate 3a). F_2 population was used as segregating population for identifying the bacterial wilt resistance gene. Artificial inculation of the plants with bacteria was done and the level of resistance was observed. The wilted plants showed positive response to bacterial ooze test (Plate 5b.). Symptoms were observed by drooping at top and yellowing of lower most leaves, followed by complete wilting started from top and death of the plants occurred within 10 days. The genotypes were classified as resistant or susceptible according to the classification of the Mew and Ho (1976). It was found that out of 200 plants, 139 (69.5 per cent) plants were susceptible to the disease and 61 (30.5 per cent) were resistant. The ratio of susceptible and resistant plants in F_2 was 3:1. This directly pointed that the resistance is governed by homozygous recessive condition.

4.2.3. Chi-square test

The total number of plants falling into different disease reaction classes (resistant and susceptible) were counted and subjected to chi-square (x^2) analysis for goodness of fit to classical Mendelian ratios and the inheritance of bacterial wilt disease incidance was revealed. The data fit a 3:1 (susceptible: resistant) ratio with x^2 probability value was · 0.58 which showed $P < 0.05$ (Table 7.). So these results suggest that resistance was encoded by recessive gene action.

Table 6. Disease reactions in parental, F_1 and F_2 generations of chillies for bacterial wilt

Sl. No.	Variety/ Generation	No. of plants artificially inoculated	No. of plants Wilted	Per cent of wilt incident	Disease reaction
1	Anugraha	60	4	6.66	R
2	Pusa Jwala	60	56	93.33	S
3	F_1	100	58	58.00	~1:1 (S:R)
4	F_2	200	139	69.5	~3:1 (S:R)

Table 7. Chi square test for segregation of resistance and susceptability in F_2 population and revealed inheritance of bacterial wilt disease

Sl. No.	Genotypes	Total no. of plants	Susceptible plants		(O-E)	$(O-E)^2$	$(O-E)^2$ /E (probabiltiy)	Mean $(O-E)^2$ /E
			Observed (O) mean	Expected (E) mean				
1	Anugraha	60	4	0	4	16	0	
2	Pusa Jwala	60	56	60	-4	-16	-0.266	-0.58
3	F_1	100	58	50	-8	-64	-1.28	
4	F_2	200	139	150	-11	-121	-0.806	

Susceptible plants

Resistant plant

a.Phenotyping of genotypes for bacterial wilt incidence

b.Ooze test for confirmation

Plate 5. Symptoms of bacterial wilt and its confirmation

4.3. Molecular characterization of chilli genotypes with AFLP assay

Molecular characterization of resistant and susceptible chilli genotypes and segregating F_2 population was done with AFLP assay for identifying the markers linked with the bacterial wilt resistance gene (Plate 6.).

4.3.1. Standardization of genomic DNA isolation

Rogers and Bendich protocol (1994) was used for genomic DNA isolation which is already reported to yield good quality DNA. The gel profile of the extracted genomic DNA from the parents, before and after the RNase treatment are presented in plate 7a and 7b respectively. The plate 7b had shown that the DNA is intact and free from RNA and protein contamination.

Along with phenotypic characterization of 200 F_2 plants for bacterial wilt resistance gene, molecular characterization was performed with AFLP markers. The genomic DNA was isolated from frozen leaves by Rogers and Bendich (1994) method. The isolated DNA had shown RNA contamination. Since the RNA may interfere with the PCR reactions. RNase treatment was given for purification of genomic DNA and purified DNA was further used for AFLP analysis (Plate 9).

4.3.2. Assessment of the quality and quantity of DNA

The quantity and quality of the isolated DNA was analyzed using electrophoresis and NanoDrop® ND-1000 spectrophotometer. In all the samples, intact clear narrow bands were observed which indicated non-degraded DNA. The ratio of UV absorbance (A260/280) ranged between 1.80-2.0 which indicated the quality of DNA was good and DNA concentration ranged between 100-300 ng/µl (Table 8).

a.Susceptible parent (Pusa Jwala)

b.Resistant parent (Anugraha)

c.F_1 Hybrid of Anugraha and Pusa Jwala

d.F_2 Progenies of Anugraha X Pusa Jwala

Plate 6. Development of F_1 and F_2 progenies

4.3.3. Bulking of F_2 DNA and Bulk Segregant Analysis (BSA)

For Bulk Segregant Analysis (BSA), DNA from susceptible and resistant F_2 plants has to be separately bulked. DNA from 9 most resistant F_2 plants were bulked and similarly, DNA from 9 most susceptible F_2 plants were also bulked.

BSA using AFLP assay was used to identify the polymorphic markers linked to the bacterial wilt resistance gene. BSA was carried out with resistant parent (Anugraha), susceptible parent (Pusa Jwala), resistant F_2 bulk, susceptible F_2 bulk and Ujwala which is the donor parent for resistance in Anugraha. AFLP primer combination EcoACT + MseCAC was reported to be specific for the Solanaceous crops by the Chromus Biotech, Bengaluru was used for BSA.
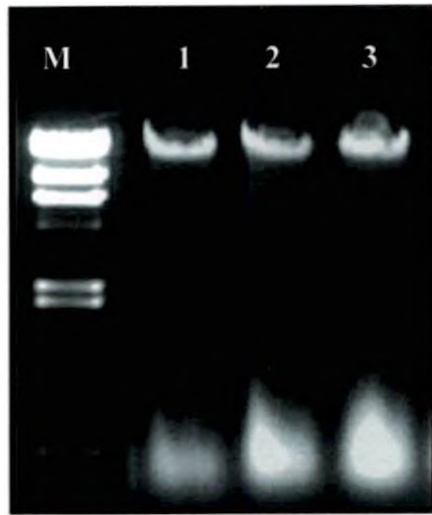
a.DNA samples before Rnase treatment
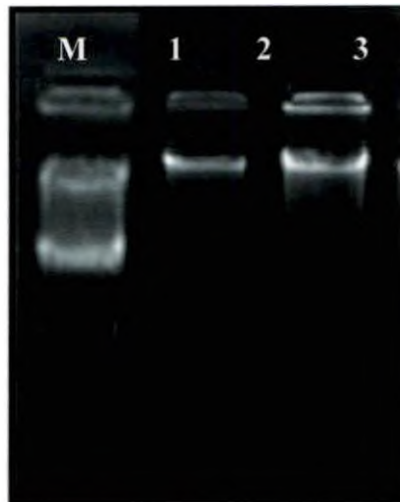
b.DNA samples after Rnase treatment

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

Lane 1:Anugraha, 2:Pusa Jwala, 3:Ujwala

Plate 7. Standardisation of DNA isolation technique
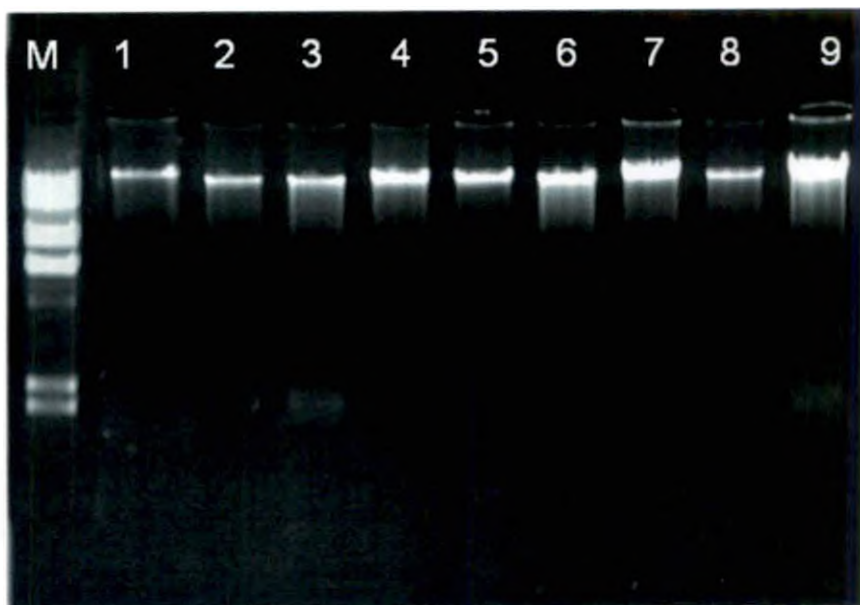
M: λDNA/*Hind*III+ *Eco*RI double digest marker

Lane 1-9: DNA samples from F$_2$ susceptible plants
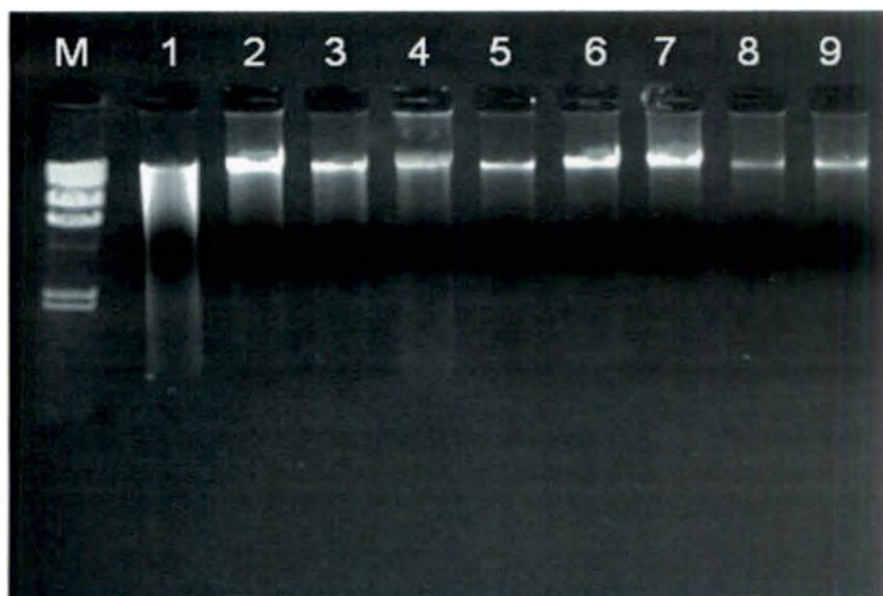
M: λDNA/*Hind*III+ *Eco*RI double digest marker

Lane 1-9: DNA samples from F$_2$ resistant plants

Plate 8. DNA samples from F$_2$ progenies

Table 8. Quality and quantity of DNA isolated from chilli genotypes as determined by nanodrop method

Genotype/ Generation	Absorbance			Quantity (ng/ul)	Quality
	A260	A280	A260/280		
Ujwala	5.523	2.863	1.93	276.17	Good
Anugraha	3.621	1.948	1.86	181.03	Good
Pusa Jwala	5.526	2.995	1.85	276.30	Good
F_2-Susceptible1	3.198	1.539	1.90	348.51	Good
Susceptible 2	3.027	1.642	1.84	151.36	Good
Susceptible 3	2.473	1.293	1.91	123.67	Good
Susceptible 4	1.556	0.795	1.96	77.79	Good
Susceptible 5	11.359	5.729	1.98	576.94	Good
Susceptible 6	2.078	1.122	1.85	103.89	Good
Susceptible 7	4.625	2.314	2.0	231.25	Good
Susceptible 8	16.930	8.225	2.06	846.49	Good
Susceptible 9	2.069	1.110	1.8	104.66	Good
F_2-Resistant 1	4.845	2.691	1.80	242.26	Good
Resistant 2	4.783	2.495	1.92	239.17	Good
Resistant 3	1.638	0.861	1.90	81.92	Good
Resistant 4	2.043	1.060	1.93	102.14	Good
Resistant 5	3.288	1.827	1.80	164.39	Good
Resistant 6	5.495	2.814	1.95	274.74	Good
Resistant 7	6.093	3.395	1.80	304.67	Good
Resistant 8	9.358	5.045	1.85	476.92	Good
Resistant 9	9.567	4.806	1.99	478.37	Good

S-Susceptible

R-Resistant

4.3.4. Analysis of selective- AFLP PCR product

AFLP-PCR product was electrophoresed using capillary electrophoresis on an ABI Prism 310 Genetic Analyzer with GeneScan™ 500 LIZ® size standard, the AFLP products of selective amplifications has generated DNA fragments in the size range of 35-500 bp. Data regarding selectively amplified DNA fragments collected using Data Collection 2.1 software (Applied Biosystems) are presented in Plate 9, 10, 11 and Table 9, 10, 11, 12, 13.

4.3.5. Analysis of amplification profiles

To analyze the AFLP markers, GeneScan 2.2 software was used and this software has measured the peaks generated by the capillary electrophoresis unit to detect the presence or absence of bands in corresponding to each marker sizes. GeneScan2.2 software has also generated a file that details the presence or absence of sizes for each marker in a Microsoft Excel format that enables the identification of polymorphism manually. Number of bands generated by the specific primer combination (EcoACT+ MseCAC) was 124 with the sizes ranging from 50 to 500 bp. Among these 124 bands, six were polymorphic for the character bacterial wilt resistance. Out of six, three polymorphic bands in the size range of 103 bp, 118 bp, and 161 bp were present in in Ujwala, resistant bulk and resistant parent Anugraha and absent in susceptible bulk and susceptible parent Pusa Jwala. In susceptible bulk and Pusa Jwala, three bands with 183 bp, 296 bp and 319 bp sizes were present and they were absent in Ujwala, resistant bulk and Anugraha.

4.3.7. Cluster analysis

Data generated by GeneScan software 2.2 was scored for the presence (1) or absence (0) of the markers in 3 parents and resistant and susceptible bulks. 124 bands generated from the primer combination under study were subjected to diversity analysis using the NtSys software. In usual diversity analyses, results from many primer combinations will be employed but in this case, the objective

of the diversity analysis was only to demonstrate and confirm the relative genetic distances between the NILs, donor parent and F_2 bulks under study, rather than the precise estimation of per cent genetic distances.

The binary data matrix was used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient for variety was calculated and used for cluster analysis using UPGMA method and a dendrogram was constructed using the software package NTSYS-PC (Rohlf, 1986). The dendrogram generated is given in Fig.1 and the details of clusters are presented in Table 14, 15. Dendrogram grouped the accessions into three main clusters. Pusa Jwala and Anugraha which are the NILs had fallen in the first cluster and they had shown 56 per cent similarity. The second cluster which was comparatively closer to the NILs was that comprising of resistant and susceptible bulks. The bulks had given the homology of 52 per cent. The donor parent Ujwala had grouped separately and formed the third cluster. These results had clearly shown that the genetic distances among the lines under study are exactly following the postulates and AFLP analysis with the large number of markers generated are capable of bringing out the comparative genetic relations even with a single primer combination.
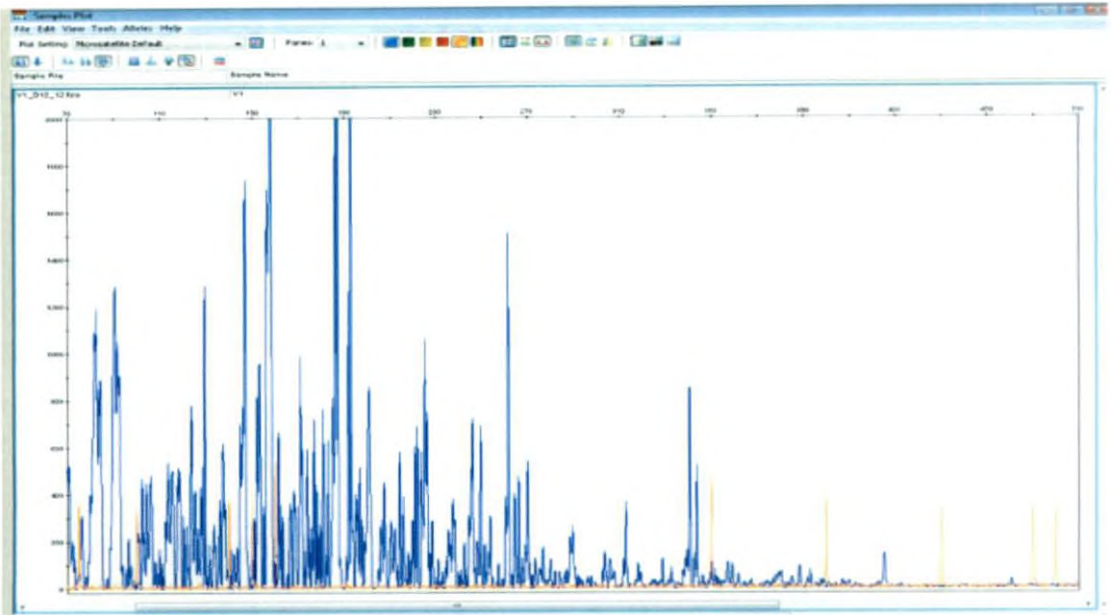
ʌʌ⋀ - Amplifid fragment of DNA, ʌʌʌʌ - GeneScan™ 500 LIZ®

AFLP peak data: Ujwala
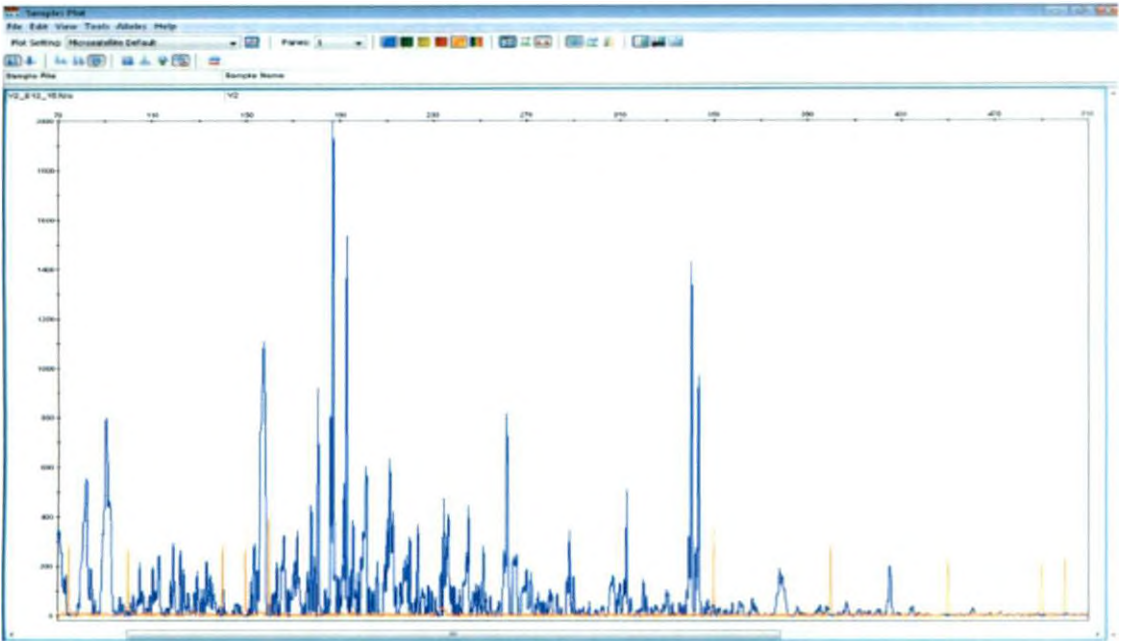
ʌʌ⋀ - Amplifid fragment of DNA, ʌʌʌʌ - GeneScan™ 500 LIZ®

AFLP peak data: Pusa Jwala

Plate 9. Electropherogram of selctive amplification by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer
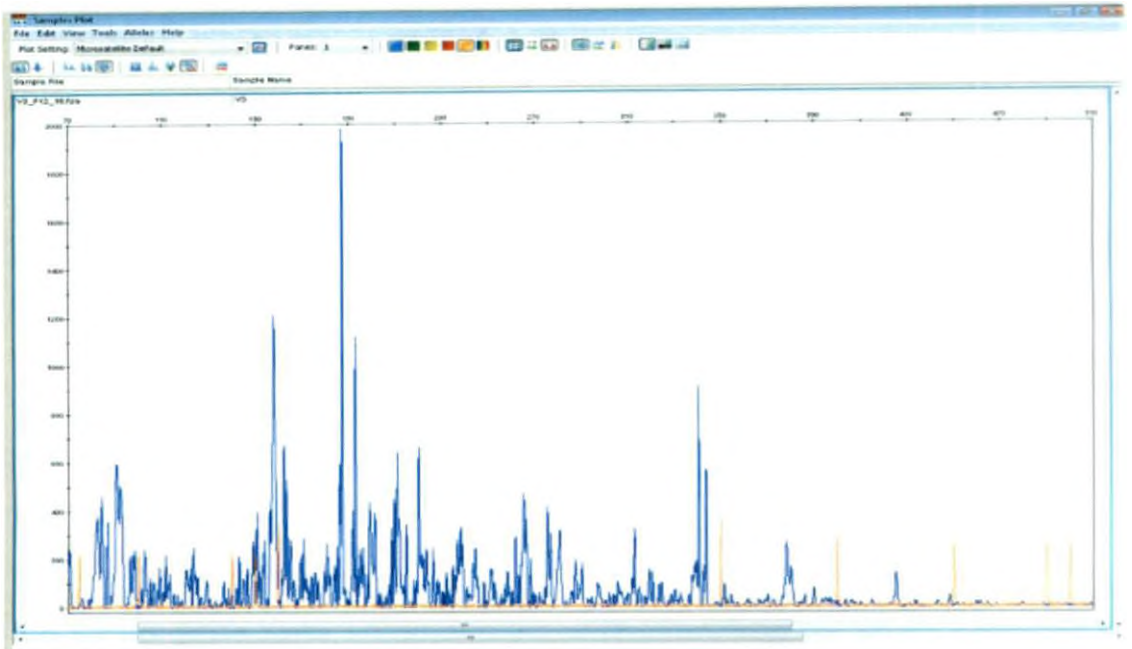
- Amplifid fragment of DNA, - GeneScan™ 500 LIZ

AFLP peak data: Susceptible Bulk
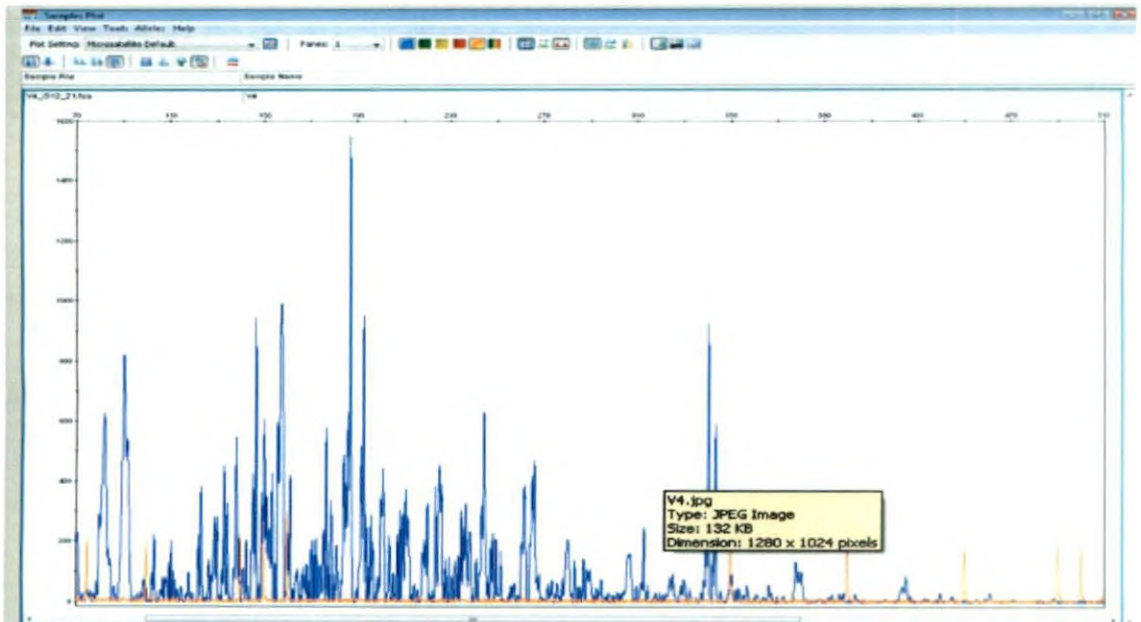
- Amplifid fragment of DNA, - GeneScan™ 500 LIZ®

AFLP peak data: Resistant Bulk

Plate 10. Electropherogram of selctive amplification by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer
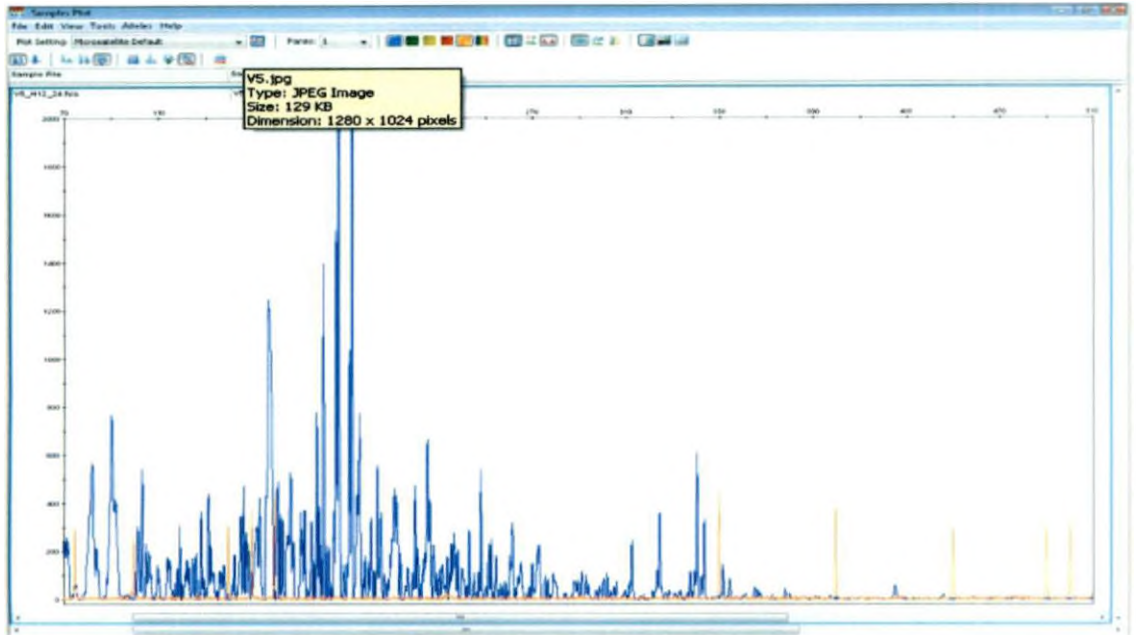
- Amplifid fragment of DNA, - GeneScan™ 500 LIZ®

AFLP peak data: Anugraha

Plate 11. Electropherogram of selctive amplification by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer
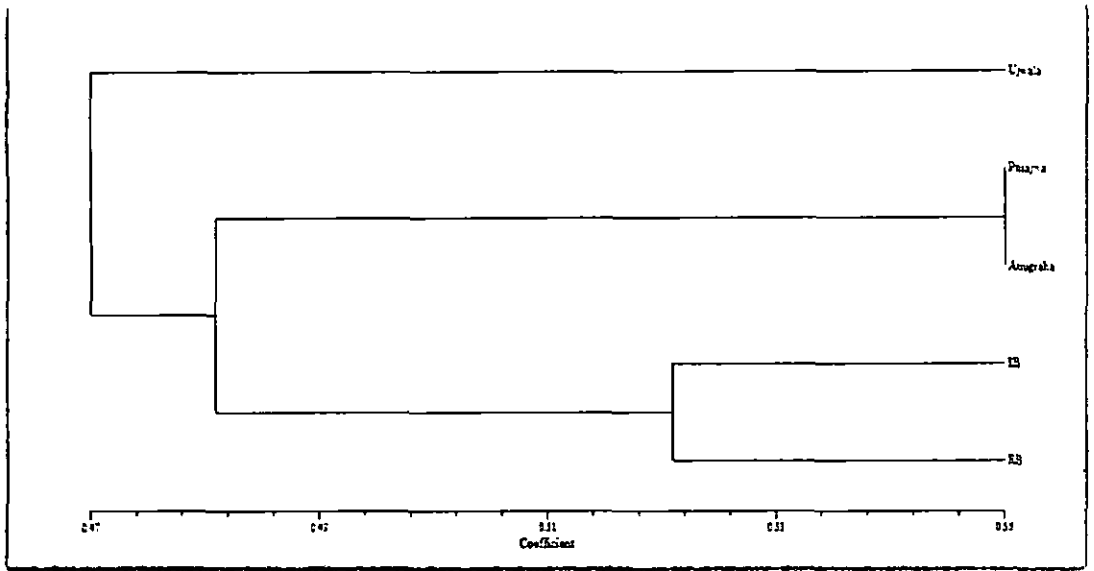
SB: F_2 Susceptible Bulk, RB: F_2 Resistant Bulk

Fig1. Dendrogram generated for chilli variety out of AFLP data using the software NTSYS

Table 9. The computer documentation of AFLP selective amplification profile
from genetic analyzer from size1 to size 27

Band Size	Ujwala	Pusa Jwala	Susceptible Bulk	Resistant Bulk	Anugraha
Size 1	55.61	54.84		55.35	55.45
Size 2				57.49	
Size 3				64.17	
Size 4	67.25	67.34	67.65	67.51	
Size 5	70.59	70.16	70.45	70.59	
Size 6					71.41
Size 7					75.13
Size 8	76.21				
Size 9	84.26	84.33	84.15		
Size 10			86.83	86.28	
Size 11			92.17		
Size 12	96.41	96.67			
Size 13			98.41		
Size 14				99.47	
Size 15	100.66				101.58
Size 16	102.25		102.49		
Size 17	**104.1**			**103.42**	**103.42**
Size 18		105.01	104.97		105
Size 19	106.21				
Size 20	107.93		108.89		
Size 21	109.77	110.54		110.66	109.99
Size 22			111.5		
Size 23	113.33	113.3	113.33		
Size 24				114.86	114.19
Size 25	115.31		115.15		
Size 26	**117.68**			**118.01**	**117.47**
Size 27		119.08			119.31

Table 10. The computer documentation of AFLP selective amplification profile from genetic analyzer from size 28 to size 54

Band size	Ujwala	Pusa Jwala	Susceptible bulk	Resistant bulk	Anugraha
Size 28			120.24		
Size 29	121.63	122.23			122.46
Size 30	123.6		123.49	123.39	
Size 31	125.18	125.5			124.95
Size 32				126.66	
Size 33	127.67				128.61
Size 34	129.38	129.3	129.22		
Size 35	131.35	131.26			131.63
Size 36	133.45	133.35		133.34	
Size 37		135.04			
Size 38	137.12		136.36		
Size 39				138.43	138.17
Size 40		140.67			
Size 41			143.06	142.79	142.8
Size 42	146.88		146.48	146.74	
Size 43			148.96		148.57
Size 44	150		150.75	150.25	
Size 45	**161.48**			**161.48**	**161.22**
Size 46		163.2			163.18
Size 47			165.02	164.43	
Size 48	166.53	166.16		166.76	166.6
Size 49	168.13			168.48	
Size 50	170.96		170.53	170.45	170.99
Size 51				172.78	172.7
Size 52	173.91				
Size 53			175.54		175.51
Size 54	176.74	176.12		176.83	

Table 11. The computer documentation of AFLP selective amplification profile from genetic analyzer from size 55 to size 81

Band size	Ujwala	Pusa Jwala	Susceptible Bulk	Resistant Bulk	anugraha
Size 55	178.71	177.84			177.83
Size 56		**183.86**	**183.61**		
Size 57	184.98			184.32	
Size 58		189.39			189.65
Size 59	194.92				
Size 60		195.76	196.29	195.83	195.99
Size 61	196.76				
Size 62			198.97		198.42
Size 63		204.47		203.9	203.66
Size 64				205.49	205.24
Size 65		206.06			206.7
Size 66	207.3				
Size 67	210.24		210.9	210.87	210.84
Size 68	211.96	211.32			
Size 69	214.16		214.67	214.53	
Size 70	215.75				216.67
Size 71	217.83		218.07		
Size 72	221.87		221.95		
Size 73		223.42	223.41	223.68	
Size 74	225.17	225.38		225.38	225.06
Size 75			226.32		
Size 76				229.65	229.31
Size 77	230.92	230.99			231.37
Size 78	232.75		232.02	232.08	
Size 79					233.43
Size 80		234.53	234.69	234.64	
Size 81		236.48	236.63	236.59	236.34

Table 12. The computer documentation of AFLP selective amplification profile from genetic analyzer from size 82 to size 107

Band size	Ujwala	Pusa Jwala	Susceptible Bulk	Resistant Bulk	Anugraha
Size 82	237.39				
Size 83			238.08	238.65	
Size 84		241.24	240.74	241.09	
Size 85	242.39				242.89
Size 86		245.01	244.37	244.37	244.95
Size 87	245.93				
Size 88	249.58	249.52		249.59	
Size 89	251.17	251.35	251.15	251.65	
Size 90					252.35
Size 91	253.73	254.39			254.4
Size 92	257.14			257.36	
Size 93		258.16	258.27		258.52
Size 94	264.2				
Size 95			268.17	268.02	
Size 96	269.91	269.82	269.97		269.77
Size 97		271.89	272.02	271.9	272.19
Size 98	273.07			273.35	
Size 99	275.02	274.8	275.39	275.65	275.58
Size 100	280	279.77	280.57	280.12	
Size 101	283.76	282.92		283.14	
Size 102			285.5	284.71	
Size 103			287.19	287.01	
Size 104		288.25			
Size 105	289.46	290.07	290.19	289.67	289.35
Size 106				294.37	
Size 107		**296.6**	**296.92**		

Table 13. The computer documentation of AFLP selective amplification profile from genetic analyzer from size108 to size 124
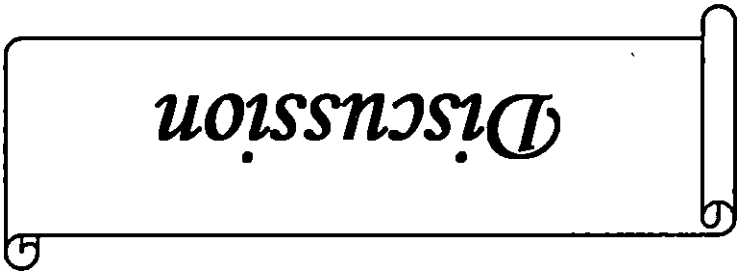
Band size	Ujwala	Pusa Jwala	Susceptible bulk	Resistant bulk	Anugraha
Size 108	305.46		305.43		305.87
Size 109	307.51	306.76		306.79	
Size 110		309.66	309.75	309.56	
Size 111		314.36	314.9	314.49	
Size 112		**319.79**	**318.97**		
Size 113			320.52		
Size 114			324.35		324.41
Size 115		329.68	330.08	329.87	
Size 116	338.68	338.58	338.68	338.5	
Size 117	351.43		351.43		351.54
Size 118	356.94			357.03	
Size 119		366.36		366.33	
Size 120	378.3	377.99	377.94	377.81	
Size 121	380.34		379.97	380.44	
Size 122		.	389.8		
Size 123				423.23	
Size 124	424.96	424.96	424.97	424.83	424.96

Table 14. Grouping of variety based on AFLP data using the softwares NTSYS

Cluster No	No. of variety in each cluster	Name of the variety
Cluster I	1	Ujwala
Cluster II	4	Pusa Jwala, Anugraha, F_2 susceptible bulk, F_2 resistant bulk

Table 15. Dissimilarity matrix of chilli variety based on the AFLP fragments

	Ujwala	Pusa Jwala	F_2 Susceptible Bulk	F_2 Resistant Bulk	Anugraha
Ujwala	1.0000				
Pusa Jwala	0.4919	1.0000			
Susceptible Bulk	0.4355	0.5242	1.0000		
Resistant Bulk	0.4919	0.5323	0.5242	1.0000	
Anugraha	0.4516	0.5565	0.4032	0.4597	1.0000

Discussion

5. DISCUSSION

Chilli or hot pepper (*Capsicum annuum* L.) is one of the most important Solanaceous vegetable crops grown worldwide. The bacterial wilt disease caused by *Ralstonia solanacearum* (Smith, 1896, Yabuuchi *et al.,* 1992) is predominant in warm humid tropical and sub-tropical regions of the world (Hayward, 1991). In India, 80-85% losses were reported in chilli due to bacterial wilt and Kerala characterized by warm, humid tropical climate and acidic soil is a hot spot for bacterial wilt which results in the 100 per cent yield losses (Mathew and Peter, 2004).

Conventional breeding involving germplasm collection, selection and heterosis breeding has resulted in the release of a number of resistant varieties. Gopalakrishnan and Peter (1991) concluded that a variety "Manjari" was resistant to bacterial wilt disease in chilli. Gopalakrishnan and Peter (1991) have reported Ujwala as highly resistant with good dry chilli yeild. Also the transgenic approach shows potential for the genetic improvement of the crop using a wide set of transgenes currently available which may confer bacterial resistance (Tripathi *et al.,* 2004).

Various genetic strategies done for reducing the yield losses from bacterial wilt in Solanaceous crops include mapping and tagging and cloning of genes for resistance with molecular markers, transfer of genes to the elite genetic background through Marker Assisted Selection (MAS), also introduction of cloned genes through transformation into elite cultivars under tissue-specific expression (Lee *et al.,* 2002).

Genetic knowledge on the inheritance of resistance is important in breeding for resistance. A limited number of recessive resistance genes have been characterized in various plant species. Study on the nature of inheritance showed that bacterial wilt resistance is monogenic and homozygous recessive (Markose,

2001). But partial resistance of pepper to bacterial wilt is also reported to be oligogenic and stable under tropical conditions (Lafortune *et al.,* 2005). So, the mode of resistance is still uncertain and the resistance is strongly influenced by environmental conditions such as temperature, pH and soil nature (Scott and Kasai, 2004).

Molecular markers like RAPD, RFLP, SSR, SCAR, CAPS, and ISSR are commonly used to characterize genetic diversity within or between populations or groups of individuals because they typically detect high levels of polymorphism (Fock *et al.,* 2000; Kumari, 2008). AFLP is highly powerful and reproducible marker suited for identifying the genes for specific characters from the large number of markers (50-200 bp) that it generates more number of polymorphic bands (Vos *et al.,* 1995).

Hence, the present study "Identification of AFLP marker linked with bacterial wilt resistance in chilli (*Capsicum annuum* L.)" was taken up. The methodology involved was bulk segregant analysis. The technical programme involved the following aspects:

1.   Development of mapping population

2.   Genetic analysis for bacterial wilt resistance and

3.   Identification of marker for bacterial wilt resistance gene from chilli genotypes with AFLP assay

The results obtained on various aspects are discussed here under.

5.1. Development of mapping population

5.1.1. Identification of genotypes for disease response

To confirm the disease resistance in Anugraha and in susceptible variety Pusa Jwala, screening was done by artificial inoculation of *R. solanacearum* with pin pricking and leaf cutting method and the symptom development was observed by ooze test. Controlled crosses were made by selecting most resistant

plant of Anugraha as the ovule parent and susceptible plant Pusa Jwala as the pollen parent to generate the F_1 seeds.

Whenever a study is designed with the objective to screen any specific gene of interest, the best strategy is to choose the Near Isogenic Lines which are differing only for the specific character. When no characterization of the gene of interest is done and sequence of the syntenic genes is not working in the concerned population, the alternate is to select the accessions differing widely in the character expression and to go for the Bulk Segregant Analysis (BSA) (Michelmore *et al.,* 1991). Ultimately the best strategy is to practice the BSA with NILs and to analyse the polymorphism using powerful and reproducible molecular marker system such as AFLP.

Kerala Agricultural University has developed bacterial wilt [*Ralstonia solanacearum* (Smith, 1896) Yabuuchi *et al.,* 1992] resistant hot pepper variety Anugraha by transferring the resistance available in the variety Ujwala into Pusa Jwala through backcrossing with Pusa Jwala as the recurrent parent (Gokulapalan *et al.,* 2004). Thus Anugraha and Pusa Jwala are Near Isogenic Lines (NILs) where, Pusa Jwala is highly susceptible and Anugraha is highly resistant for the bacterial wilt disease.

5.1.2. Induction of the disease symptoms

Artificial inoculation was done for understanding the susceptibility and resistance in parents, F_1 and F_2 populations using 30 days old seedlings. Three methods of inoculation were used viz., pin pricking, leaf cutting and soil drenching with stem wounding at collar region. The susceptible genotypes showed wilting within 5 days after inoculation.

In this study, plants have been inoculated 3-4 times as a precautionary measure only to ensure pathogen entry in 30 days old seedling which has thick

cortical cells compared to seedlings at early growth stage. Based on the results, leag cutting and pin pricking methods were chosen for further screening.

The choice of the method is very much dependent on the environmental conditions and many researchers have adopted different methodologies for this purpose. However, it was difficult to draw solid conclusions from the available reviews as which could be the best methodology for screening. Winsted and Kelman (1952) had reported the wilting in tomato within 5 days using stem-puncture and leaf cutting method. In this study leaf cutting and pin pricking methods were best for induction symptoms for bacterial wilt disease. Karmannil (2007) and Ragina (2009) also used leaf cutting in tomato to induce 100 per cent wilt incidence one week after inoculation. However, Hoque and Mansfield (2005) and Di et al. (1991) used pin pricking method to induce disease incidence in rice for bacterial leaf blight and observed wilting after 10 days. This once again confirms that according to crops, stage of crop and the prevailing environments, the inoculation strategy has to be standardized.

5.1.2. Screening of parental generation

The parental generation was screened artificially in open field and scoring of genotypes as resistant/susceptible was done by Mew and Ho (1976) system. Pusa Jwala plants showed 93.33 per cent of wilt incidence within one month after inoculation whereas Anugraha showed 6.66 per cent of wilt incidence. Gokulapalan et al. (2004) reported Anugraha is resistant to bacterial wilt which was also observed in present study.

5.1.3. Screening the F_1 generation

To verify gene action, F_1 plants were raised in open field and screening was done by artificial inoculation of R. solanacearum and per cent of wilt incidence was calculated. Out of 100 plants screened, 58 were found to be susceptible and remaining 42 plants were resistant. The chi-square analysis had shown that the population follows 1:1 ratio for the resistance. Markose et al.,

(2001) has reported that resistance to bacterial wilt is offered by homozygous recessive condition that is single gene is responsible for resistant character. So among all F_1 plants of the crossed resistant Anugraha and susceptible Pusa Jwala are supposed to be susceptible to bacterial disease. But the results in the present study were not in accordance with this report and the deviation from the previous report may be due to the heterozygous allelic (Rr) condition of the susceptible parent. . When the gene action for resistance is homozygous recessive (rr), if the susceptible parent is homozygous for the allele (RR), all the F_1 plants derived from the cross of resistant and susceptible plants will have the uniform genotype Rr and we can expect the disease reaction of complete susceptibility. Whereas in this case the susceptible plant selected had Rr allelic situation, leading to two distinct genotypes in F_1, 50% plants with Rr (susceptible) and 50% plants with rr (resistant) alleles.

Since only the first category of susceptible F_1 plants (Rr) will only produce the segregating population in F_2, and if we raise these category directly in the field for sefing and F_2 seed harvest, there is every chance that these plants will wilt before the fruit maturity. Hence, 10 F_1 plants were raised in pots, selfed and F_2 seeds harvested separately. After harvesting F_2 seeds, all pots were screened with bacteria and susceptible plants were identified and F_2 seeds were collected from the first wilted F_1 plant was advance to F_2 generation.

5.1.4. Screening the F_2 generation

Segregating population of 200 F_2 plants were raised in open field and screening through artificial inoculation was done for confirmation of disease resistance. F_2 plants are reported to offer certain advantages over other mapping population (DH lines, NIL's etc) because segregation is fixed (Benjamin and Burr, 1999). However plants from such segregating population have to be grouped according to accurate phenotypic screening of the trait.

Grimaul *et al.,* (2008) have previously reported that in tomato, resistance to *P. solanacearum* in F_2 generation of resistant variety Hawaii 7996 and susceptible Floradel was monogenic and dominant. The F_1 plants did not wilt and a significant 3:1 segregation ratio was obtained for non-wilting: wilting in F_2. In line with the results from the present study, Markose (1996) isolated bacterial wilt pathogen *Pseudomonas solanacearum* from the infected chilli plants and established its pathogenecity and showed that the resistance to this disease is a monogenic and incompletely dominant type of inheritance. However, the bacterial wilt resistance in the sweet pepper variety 'Mie-Midori' showed incomplete dominance and at least two genes were involved in resistance (Matsunaga *et al.,* 1998).

Hao *et al.* (2009) reported that the inheritance of bacterial wilt resistance in eggplant was controlled by a single dominant gene following the Mendelian inheritance. They used the F_1 and F_2 BC_1 segregating populations derived by crossing highly resistant to bacterial wilt *Ralstonia solanacearum* bacteria Race1 (E-31) and susceptible (E-32). Study in brinjal conducted at Kerala Agriculture University has revealed that resistant F_1s could be developed by crossing resistant parents which indicates recessive mode of inheritance of bacterial wilt resistance (KAU, 1989).

At Kerala Agricultural University, Peter *et al.* (1984) made crosses of two hot pepper lines viz., 'Manjiri' and 'Pant C-1' with five sweet peppers. All the F_1 were susceptible or moderately susceptible indicating the recessive nature of inheritance of resistance to bacterial wilt. Markose (1996) studied the inheritance of bacterial wilt resistance using resistant variety Ujwala and susceptible variety Pusa Jwala by developing six generation of P_1, P_2, F_1, F_2, BC_1 and BC_2. Performance of the six generation showed the resistance in Ujwala is monogenically inherited and is incompletely dominant.

In *Arabidopsis*, Deslandes *et al.* (2002) reported two genes RRS1-R which is a recessive and RRS1-S which is a dominant, conferring resistance to *R. solanacearum*. Then Monma and Sakata (1993) also reported that the bacterial wilt resistance was partially recessive as there was incomplete dominance towards susceptibility. In tomato, Sreelathakumary (1983) reported that a complementary and hypostatic type of digenic recessive gene responsible for resistance to bacterial wilt. In line with present findings, Rajan (1985) reported bacterial wilt resistance gene in tomato variety 'Sakthi' is monogenic and incompletely dominant.

From these discussions, it is clear that the gene action for bacterial wilt resistance varies widely in each crop. Though belong to the same family of Solanaceae, capsicum, brinjal and tomato have different gene actions. Similarly, the *Capsicum annuum* at different parts of the world shows varying gene action and it can not be generalized for this genus. Many studies point out the fact that the gene action for resistance in the chilli varieties released by Kerala Agricultural University is monogenic recessive.

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 identifying marker. The DNA of nine highly susceptible and nine most resistant genotypes were bulked. F_2 individuals along with parents were examined by marker analysis and polymorphism between the Ujwala, susceptible bulk, Pusa Jwala, resistant bulk and Anugraha were revealed by selective primer combinations. Obtained polymorphism was associated with the resistance with comparable to previous reports in *Capsicum* (Miao *et al.,* 2009).

5.3. Bulk Segregant Analysis (BSA) with AFLP assay

Molecular characterisation of the susceptible and resistant chilli genotypes and their F_2 susceptible and resistant population was carried out with the

objective of identifying marker for bacterial wilt resistance. A powerful and reproducible molecular marker system AFLP (Amplified Fragment Length Polymorphism) was used for the characterization.

5.3.1. Genomic DNA used for molecular characterization

Isolation of good quality genomic DNA is one of the most important pre-requisites for doing AFLP analysis. The procedure reported by Rogers and Bendich (1994) for the extraction of nucleic acid was used for isolation of genomic DNA from chilli with slight modifications. Tender leaves were chosen for DNA extraction in all the cases. The method suggested by Rogers and Bendich (1994) was the best for the isolation of the genomic DNA from chilli since distinct bands without shearing were obtained. Recovery of DNA was also high. Tender leaves contain actively dividing cells with lesser concentration of extra nuclear materials like carbohydrate, fats, protein, oil and other metabolites which interfere with the isolation of nucleic acid. Fu *et al.* (2004) also reported that the use of young leaves was best for DNA isolation for AFLP assay. So in this study also Rogers and Bendich (1994) protocol was used with some modification made by giving Rnase treatment after DNA extraction.

5.3.2. Bulk Segregant Analysis

BSA was carried out with the DNA from resistant parent (Anugraha), susceptible parent (Pusa Jwala), resistant bulk (9 most resistant plants from F_2), susceptible bulk (9 susceptible plants from F_2) and Ujwala which is the donor parent for resistance in Anugraha. The segregating population of F_2 plants was originated from the most susceptible F_1 plant by selfing. The specific AFLP primer combination EcoACT+ MseCAC and their corresponding adapters, which are reported suitable for the Solanaceous plants by the Chromus Biotech Pvt. Ltd., Bangalore was used for BSA. EcoACT+ MseCAC produced polymorphism in size from 50 to 500 bp with 124 bands.

BSA involves comparing two pooled DNA samples of individuals from a segregating population originating from a single cross and the advantages of BSA over other genetical techniques for gene 'tagging', is well demonstrated (Michelmore *et al.*, 1991). Within each 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 allelic frequency with linked loci and generally it is recommended that the number of plants from F_2 population used for DNA bulking should not be less than 5 or should not exceed 10 (Michelmore *et al.*, 1991).

5.3.3. AFLP (Amplified Fragment Length Polymorphism) analysis

In this study, the AFLP method was demonstrated to be effective in detecting candidate markers associated with bacterial wilt resistance genes. Previously, RAPD method has been attempted to detect markers for this gene in chilli (Kumari, 2008). Even after screening more than 100 primers in BSA, these attempts were not successful. RAPD assay usually generates 4-10 markers in each primer and the chance of obtaining the polymorphic bands is comparatively lesser and further, the reproducibility of RAPD markers is a question. The AFLP assay involves two levels of restriction digestion and hence the chance of generating the polymorphic bands originating from each allele will be more. The number of polymorphic bands generated varies with the number of restriction sites present is the genic alleles or the linked marker locations. The general ability of the AFLP method to detect these polymorphic markers again depends on the capability of the electrophoretic technique to detect even the small fragments of DNA. This gives a higher chance to design site specific primers, which is a further advantage of the simplified AFLP method. Usually, the electrophoretic methodology followed AFLP assay is PAGE (Polyacrylamide Gel Electrophoresis) which is comparatively better over the agarose gel electrophoresis followed for RAPD. In the present study, it was decided to carry out the most powerful electrophoretic methodology - capillary electrophoresis

which got the capability to separate even the smaller fragments with the size of only 50bp.

Polanco *et al.* (2005) have developed a method that allows for isolation and cloning of specific AFLP markers obtained with a laser-induced fluorescence capillary electrophoresis system. This procedure has been tested on five *Arabidopsis thaliana* polymorphic AFLP markers, and the nucleotide sequences obtained from these cloned markers were identified and located in the *Arabidopsis* genome.

Selectively amplified AFLP markers were separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer with GeneScan™ 500 LIZ® Size standard in the 35-500 nucleotides range. The standard was fifth dye-labeled for the reproducible sizing of fragment data analysis and provided 16 single-stranded labeled fragment of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 nucleotides. The sizing curve generated from these fragments made the standard ideal for a variety of fragment analysis applications such as microsatellites, and Relative Fluorescent Quantification. Each of the DNA fragments is labeled with the LIZ® fluorophore which resulted in a single peak when run under denaturing conditions.

5.3.4. Analysis of amplification profiles

Although many monomorphic bands were observed among Ujwala, Pusa Jwala, susceptible bulk, resistant bulk and Anugraha, polymorphic bands were also abundant. A total of 124 bands ranging in size approximatly 50 to 500 bp were obtained by using the Solanaceae specific EcoACT+ MseCAC primer combination. Three polymorphic bands sized 103 bp, 118 bp, 161 bp were identified in resistant lines which were absent in susceptible lines. In susceptible lines, three bands with 183 bp, 296 bp and 319 bp sizes were present and they were absent in resistant lines.

Lee *et al.,* (2010) has developed DNA markers linked to the GMS (Genic Male Sterility) ms3 gene in a segregating population using bulked segregant analysis (BSA) and amplified fragment length polymorphism (AFLP) techniques. Three AFLP markers E-AGG/M-CCC (276 bp), E-AGC/M-CTT (178 bp), and E-CAG/M-TGC (204 bp) were identified as tightly linked to the ms3 locus. Zhang *et al.* (2013) identified AFLP markers 246 bp (E-ACC/M-CAG) in treated fertile population as tightly linked to the drought stress gene and 357 bp (E-TGG/M-CTC) AFLP markers in a treated sterile population that is closely associated with the drought stress gene in *Salvia miltiorrhiza* Bunge.

Miao *et al.* (2009) also identified two AFLP markers using the primer combination E-AAG/M-CAT200 and E-AAG/M-CAT300 which were linked to bacterial wilt resistance in tomato.

5.3.5. Genetic distance between chilli varieties based on AFLP data

Estimation of genetic distances is an important application of molecular markers in crop plants and AFLP is also proven very effective in this. Usually for computing the genetic distance among accessions exactly, profiles of at least 10 primer combinations should be combined. Krishnamurthy *et al.,* 2013 reported the morphological and amplified fragment length polymorphism (AFLP) marker-based genetic diversity, to estimate mid-parent heterosis and to study the possible limits of the parental divergence for the occurrence of heterosis for yield and its contributing traits in chilli hybrids.The 8 AFLP primer combinations generated a total of 335 amplicons, of which Eco-AGC and Mse-GCT primers showed the highest percentage of polymorphic information content (PIC) of 94. Hence, these AFLP primer combinations are useful for analysis of divergence in chilli.

In the present study, the specific primer combination itself was sufficient to meet the objective of identifying the marker linked to bacterial wilt resistance and hence no further attempts with more primer combinations was necessary. However, since the primer combination has generated as much as 124 bands,

attempt was also made to roughly demonstrate once again that the NILs are genetically more related against the donor parent and the bulk. Computing the genetic distances using Cluster analysis was done using the Unpaired Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis and dendrogram was constucted by Neighbor Joining (Saitou and Nei, 1987).The results from the analysis has shown the results in line with the assumptions. The use of the dendrogram generated was only confirmatory and the distances shown by the software were not taken into consideration or those results were not used or recommended for use in subsequent studies because it was generated from a single primer combination analysis.

5.4 Possible applications of AFLP in Marker Assisted Selection (MAS)

Breeders want to incorporate agronomically interesting resistance traits in their breeding material. Marker assisted selection is a technique that can facilitate this process by the selection of genes that control traits of interest such as disease resistance. PCR based markers for *Sw-5* gene of tomato spotted wilt virus resistant have been used in tomato MAS (Shi *et al.,* 2011). In the context of MAS, E-ACT/M-CAC AFLP marker can be effectively utilized for tracing favorable allele(s) (dominant or recessive) across generations and identifying the most suitable individual(s) among the segregating progeny based on allelic composition across a part or the entire genome.

5.5. Future line of work

The detection of reproducible molecular markers for identifying the resistance gene bearing plants in the breeding population was a challenge till date. The previous attempts Kumari (2008) had used RAPD marker to identify bacterial wilt resistance gene in chilli (*Capsicum annuum* L.) itself but compared to RAPD, AFLP is highly specific for that which showed specific band for bacterial wilt resistance gene. The RAPD primers OPAB-08, OPA-09 and OPG-07 were used for development of SCAR markers from diseased soil and plant sample. One Race 2 specific SCAR marker, RS-R2 and two species specific

SCAR markers, RS-1 and RS-2 were developed. RS-R2 showed a unique 600 bp size band in Race 2 isolates, which was found absent in isolates of Race 1 and 3. Unique 920 bp and 900 bp size amplicons were observed with RS-1 and RS-2 SCAR primer respectively in all 114 isolates of *R. Solanacearum* (Gund, 2010).

The present study had revealed 6 markers which could be directly used in marker assisted selection. Since AFLP is highly reproducible, these markers could be equally potential to the SCAR markers. However, the cost involved in the AFLP and the capillary electrophoresis may be comparatively higher and hence for the simplification of the process, the following works in this line are proposed

1. Isolate mRNA from resistant and susceptible genotypes after artificial inoculation with a pathogen, synthesise cDNA and go for genome analysis through subtractive hybridization.

2. Further validation of these 6 markers could be done using the DNA from other susceptible and resistant chilli/ tomato/ eggplant lines since we expect high level gene synteny within the family

3. These selective amplification product could also be run on denaturing PAGE and polymorphic markers may be eluted, cloned and sequenced to get the sequence, further to develop the SCAR primers.

Summary

6. SUMMARY

The study entitled "Identification of AFLP marker linked with bacterial wilt resistance in chilli (*Capsicum annuum* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2011-2013. The main objective of the study was to identify the AFLP marker linked with bacterial wilt resistance in chilli (*Capsicum annuum* L.). The genotypes used for the study were resistant varieties Ujwala, Anugraha, susceptible variety Pusa Jwala and F_2 progenies of the cross Anugraha x Pusa Jwala.

The salient finding of the study are summarized below:

1. The seedlings Anugraha and Pusa Jwala were subjected to artificial inoculation through leaf cutting as well as stem puncturing methods to confirm the resistance/susceptible to bacterial wilt caused by *Ralstonia solanacearum*. Resistant parent Anugraha had shown 6.66 per cent of wilt incidence and susceptible parent Pusa Jwala had 93.33 per cent of wilt incidence. F_1 plants were raised by controlled crossing of resistant variety Anugraha as the ovule parent and susceptible variety Pusa Jwala as the pollen parent. Single susceptible F_1 plant was selfed under sterile pot culture and segregating F_2 progenies for bacterial wilt resistance were raised.

2. From the observations on the disease response in the F_1 and F_2 segregating population, the gene action was identified to be monogenically recessive. This was further confirmed through the chi-square analysis.

3. The protocol suggested by Rogers and Benedich (1994) was found to be best for the genomic DNA isolation from chilli. High quality DNA was isolated, quantified using Nanodrop spectrophotometer, evalaued using AGE and used for AFLP. Bulk Segregant Analysis (BSA) was done with DNA of resistant parent Anugraha,

susceptible parent Pusa Jwala, donor of resistance gene Ujwala, bulked DNA from 9 most susceptible F_2 plants and bulked DNA from 9 most resistant F_2 plants.

4. Specific AFLP primer combination recommended for solanaceous crops by Chromus Biotech, Bangalore, India *Eco*ACT (5'-CTCGTAGACTGCGTACCAA TTC ACT-3') + *Mse*CAC5'- (GACGATGAGTCCTGAGTAACAC-3') was used for BSA. The sequence of the adapters *Eco*A1 (5'-CTCGTAGACTGCGTACC-3') and *Eco*A2 (5'-AATTGGTACGCAGTCTAC-3') also *Mse*A1 (5'-GACGATGAGTCC TGAG-3') and *Mse*A2 (5'-TACTCAGGACTCAT-3') were used in AFLP analysis.

5. The products of selective AFLP amplifications were separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer with GeneScan™ 500 LIZ® size standard. Data regarding selectively amplified DNA fragments were collected by a computer connected to the ABI Prism 310 that was running Data Collection 2.1 software.

6. A total of 124 bands ranging in size from 50 to 500 bp were obtained and were manually analysed for the polymorphism for bacterial wilt resistance. From the analysis, six polymorphic bands were identified. Out of them, three polymorphic bands with 103 bp, 117 bp and 161 bp were present in donor parent Ujwala, resistant F_2 bulk and resistant parent Anugraha and absent in susceptible F_2 bulk and susceptible parent Pusa Jwala.

7. The other three polymorphic bands sized 186 bp, 296 bp and 319 bp were present in susceptible F_2 bulk and susceptible parent Pusa Jwala and absent in donor parent Ujwala, resistant F_2 bulk and resistant parent Anugraha.

Since AFLP is a reproducible marker system, these markers could equally useful as SCAR markers for the use in MAS and could be directly employed for identifying the resistant segregants in a breeding population.

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Annexures

List of Laboratory Equipment Used For the Study

Spectrophotometer   Spectronic Genesys-5, Spectronic Instrument USA

Horizontal electrophoresis system   Biorad

Refrigerated centrifuge   Kubota, Japan

Thermal cycler   Mastercycler personal, Eppendorf

Capillary electrophoresis system   Chromous Biotech, banglor

Gel documentation system   BIO-RAD Imaging system

ABI genetic analyzer   Chromous Biotech, banglor

Rogers and Bendich (CTAB) method

1. 2X CTAB extraction buffer

-CTAB (2%, v/v)

-100mM Tris (pH8)

-20mM EDTA (pH8)

-1.4M NaCl

-1% PVP

2. 10%CTAB solution:

-10% CTAB (w/v)

-0.7M NaCl

3. TE buffer:

-10mM Tris (pH8)

-1mM EDTA (pH8)

Composition of buffers and dyes used for agarose gel electrophoresis

1. **TAE Buffer 50X –**
   - 242 g Tris base
   - 57.1 ml glacial acetic acid
   - 100 ml 0.5 M EDTA (pH 8.0)

2. **TBE buffer 10X (for 1 litre)**

   -54g Tris base

   -27.5g boric acid

   -20ml 0.5M EDTA (pH8)

3. **Loading Dye (6X)**
   - 0.25% Bromophenol blue
   - 0.25% Xylene cyanol
   - 30% Glycerol in water

4. **Ethidium bromide**

   - The dye was prepared as a stock solution of 1mg/ml in water and was stored at room temperature in a dark bottle.

Composition of reagents used for AFLP analysis (Chromous Biotech)

Components	10 reactions
10X assay buffer (digestion)	50 ul
Sample of genomic DNA	10 ul
EcoRI (10U/ul)	5 ul
Msei (10U/ul)	5 ul
2X QLB	300 ul
EcoRI adapter (100ng/ul)	5 ul
MseI adapter (100ng/ul)	50 ul
Quick ligase	10 ul
Preamp primer mix	20 ul
10X chromoTaq assay buffer	50 ul
dNTP mix (2.5mM each)	40 ul
ChromoTaq DNA polymerase (3U/ul)	10 ul
Selective primer mix	20 ul
Water	1 ml

IDENTIFICATION OF AFLP MARKER LINKED WITH BACTERIAL WILT RESISTANCE IN CHILLI (*Capsicum annuum* L.)

By

THAKUR PRANITA PRABHAKARRAO
(2011-11-109)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the

requirement for the degree of

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ABSTRACT

Chilli is one of the most important condiments in India and our country is the largest producer, contributing 25% of the world production. During 2010-11, India produced 0.8 mt of dry chilli from an area of 0.93 mha. Bacterial wilt (caused by *Ralstonia solanacearum*) is a major reason for the lower productivity of this crop, causing up to complete losses under severe infections.

The study entitled "Identification of AFLP marker linked with bacterial wilt resistance in chilli (*Capsicum annuum* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture during the period 2011-2013. The objective of the study was to identify AFLP marker linked with bacterial wilt resistance in chilli (*Capsicum annuum* L.).

Three chilli genotypes Ujwala, Anugraha and Pusa Jwala and their progenies were used in the study. Anugraha is a chilli variety with resistance to bacterial wilt disease. Pusa Jwala is the near isogenic line (NIL) of Anugraha, both differing only in the gene for resistance to bacterial wilt. Ujwala was the donor parent for resistance while developing Anugraha from Pusa Jwala through back crossing programme.

Pusa Jwala and Anugraha varieties were screened in open field with artificial inoculation to confirm the disease reaction. Highly resistant Anugraha plant was crossed with the pollen from most susceptible Pusa Jwala plant. The F_1 seeds were harvested and this generation was field screened to observe the disease reaction. Previous report on bacterial wilt resistance points to monogenic homozygous recessive condition for resistance. Accordingly, all the F_1 plants are supposed to be susceptible; but our screening had shown the resistance in F_1 generation to be nearly 50 per cent. The reason for the deviation from the expected ratio is attributed to the selection of a heterozygous (Rr) plant as male

parent. Further the F_1 plants were raised in pots, selfed seeds were harvested and further the pots were infected with bacteria and F_2 seeds harvested from susceptible F_1s were used for raising the F_2 population. F_2 population was used as segregating population for generating the susceptible and resistant bulks in bulk segregant analysis (BSA) using AFLP method.

Screening of 200 F_2 plants was done along with parents Anugraha and Pusa Jwala using leaf cutting and pin pricking methods. F_2 segregating plants have shown 69.5 per cent wilt incidence, pointing to ~3:1 ratio, confirming that the resistance is governed by homozygous recessive condition.

DNA extraction was done from parents and all the 200 F_2 plants and Ujwala by CTAB method (Rogers and Bendich, 1994). Good quality of DNA with UV absorbance ratio (A260/280) ranged 1.8- 2.0 were used for further AFLP analysis. DNA from 9 highly susceptible and 9 highly resistant F_2 plants was separately pooled for developing bulk. BSA was carried out with AFLP analysis the DNA using resistant parent (Anugraha), susceptible parent (Pusa Jwala), resistance donor (Ujwala), F_2 resistant bulk and F_2 susceptible bulk.

The AFLP was performed using the standard kit provided by Chromus Biotech, Bangalore, following the restriction digestions using frequent and rare cutters, adapter ligation, pre-amplification and selective amplification using the primer combination EcoACT+ MseCAC. The amplified PCR products were separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer along with GeneScan™ 500 LIZ® size standard and data generated were collected by Data Collection 2.1 software. Totally, 124 bands ranging 50-500bp were amplified. Among them, three polymorphic bands with 103, 118 and 161 bp linked with the resistance allele and three polymorphic bands with 183, 296 and 319 bp linked with susceptible allele were identified. Due to the repeatable nature of AFLP, these 6 markers could be directly employed in MAS breeding programmes.