PHENOTYPING AND IDENTIFICATION OF SSR MARKER LINKED TO BACTERIAL WILT IN BRINJAL

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DEPARTMENT OF PLANT BIOTECHNOLOGY

UNIVERSITY OF AGRICULTURAL SCIENCES

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Thesis submitted to the

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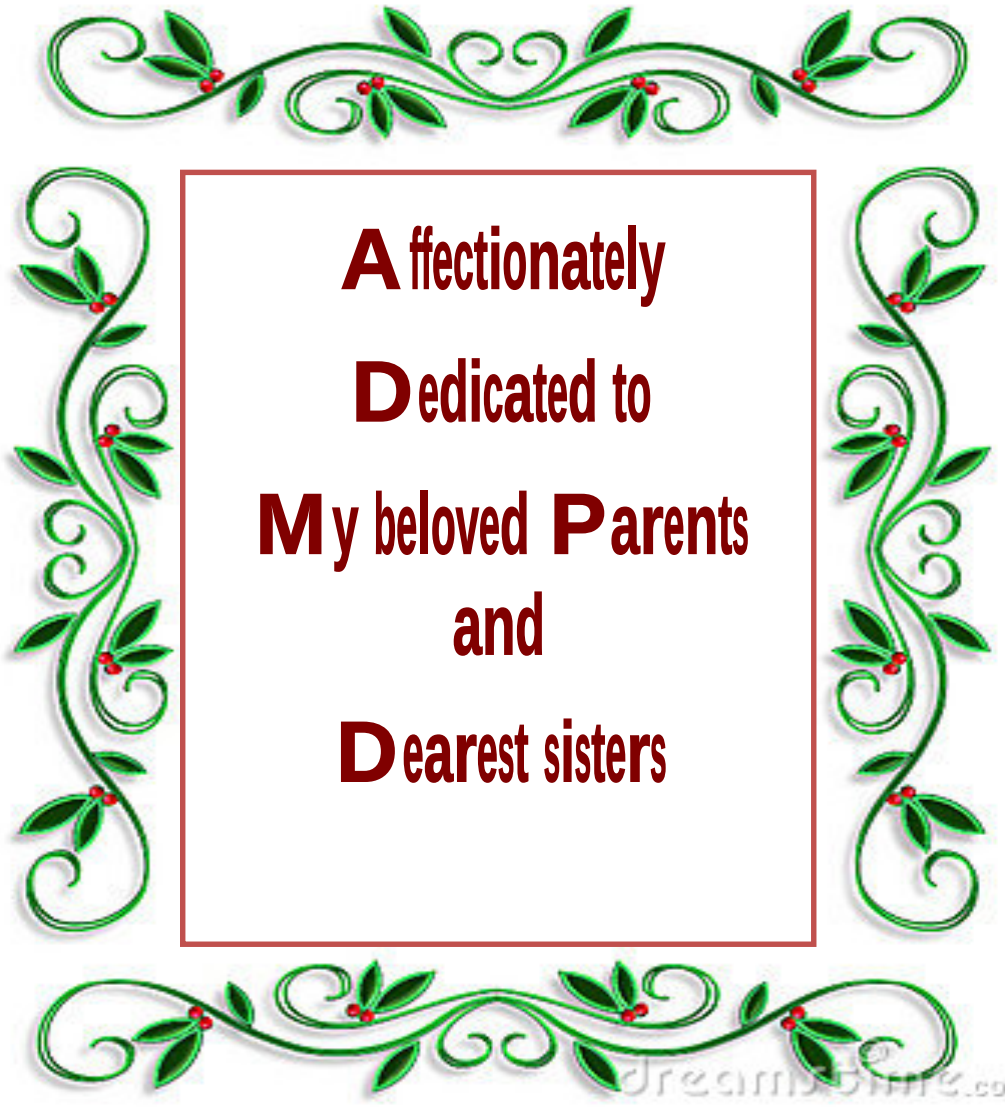
Affectionately
Dedicated to
My beloved Parents
and
Dearest sisters

DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU-560065

CERTIFICATE

This is to certify that the thesis entitled "**PHENOTYPING AND IDENTIFICATION OF SSR MARKER LINKED TO BACTERIAL WILT IN BRINJAL**" submitted by **Ms. MUKTHA, R., ID. No. PAL 0230** in partial fulfilment of the requirement for the degree of **MASTER OF SCIENCE (Agriculture)** in **PLANT BIOTECHNOLOGY** to the University of Agricultural Sciences, Bengaluru, is a record of bonafide research work done by her during the period of her study in this University under my guidance and supervision and that no part of the thesis has previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar titles.

Bengaluru,
July, 2012

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Bengaluru,
July, 2012 *MUKTHA, R.*

Phenotyping and Identification of SSR Markers Linked to Bacterial Wilt in Brinjal.

ABSTRACT

Brinjal is an important vegetable in India and is grown throughout the tropical, subtropical and warm temperate regions of the world. Bacterial wilt caused by *Ralstonia Solanacearum* is a severe soil borne vascular disease commonly occurring in the warm, humid tropics. An investigation was conducted to identify SSR markers linked to bacterial wilt resistance in brinjal in the F_2 mapping population developed by the cross between a bacterial wilt resistant variety IIHR 500A with bacterial wilt susceptible variety IIHR 575. 310 F_2 plants were phenotyped by artificial root infection with *Ralstonia Solanacearum* L.(3:1) ratio of resistance- susceptible was observed in the F_2 and the genetic ratio indicated the resistance trait controlled by a single dominant gene.

39 EST-SSRs, 167 genomic SSRs and 71 tomato *Ralstonia* linked QTL SSRs were used to genotype parents, F_2 population through bulk segregant analysis(BSA). 15 genomic SSRs and 6 tomato *Ralstonia* linked QTL SSRs had shown polymorphism in the parental lines and were further used to screen the mapping population. No polymorphism was observed in the F_2 population. Thus, no marker linked to Bacterial wilt resistance in brinjal was identified in the present F_2 population under study.

Further, linkage analyses are to be performed to identify the genomic SSRs using more primers that are linked to the resistance trait in a larger population and integrated into a molecular marker associated breeding programme of eggplant to breed bacterial wilt resistant varieties.

Signature of the student — Signature of the Major Advisor

Muktha, R. — **Dr C. Aswath**

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INTRODUCTION

I. INTRODUCTION

The eggplant or brinjal or aubergine (*Solanum melongena* L.) represents the non-tuberous group of *Solanum* species (Narasimha Rao, 1979). Brinjal is an autogamous diploid with 12 haploid chromosomes (2*n* = 24). Brinjal is an often cross pollinated and annual herbaceous plant. It has considerable economic importance in Asia, Africa, and the subtropics (India, Central America), and in some warm temperate regions (Mediterranean area, South of the USA) (Sihachakr *et al*., 1993).

Eggplant is a native of the Indian subcontinent and India is the probable origin of eggplant (Bhandari, 1951). Vavilov (1931) felt that its centre of origin was in the Indo-Burma region. Its close relatives: *Solanum aethiopicum* (scarlet eggplant) and *Solanum macrocarpon* (Gboma eggplant) are of African origin. A wild type with many small fruits, sometimes called as S.*melongena* var. *insanum,* is found on the Bengal plains of India. Various forms, colours and shapes of eggplant are found throughout South-east Asia, suggesting that this area is an important centre of variation and possibly of origin.

India is the second largest producer of brinjal in the world after China and is followed by Egypt and Turkey (FAO Website, 2010). In India, brinjal is grown over an area of 6.12 million ha with an estimated annual production of 10.563 million metric tonnes with a productivity of 17.3 metric tonnes per ha. West Bengal (26.4%) is the leading brinjal producing state in India followed by Orissa (20.2%) and Bihar (11.4%) (National Horticulture Board, 2010).

Eggplant has been divided into three main types, egg shaped *S.melongena*, var. *esculentum* (Common eggplant), long slender shaped var. *serpentium* and dwarf type var. *depressum*. There are also many wild species of eggplant that carry many important genes. Wild species

show resistance to many important diseases and pests that affect commercial eggplant production (Chen *et al.,* 2000). Cytological studies on eggplant species have been instrumental in classification of the plant. Although the basic chromosomal number (n=12) is same in all the varieties and species, the cytology and chromosome number of many diploids, autotetraploids and haploids have been well studied in eggplant. *Solanum* is one of the largest genera of vascular plants having more than 1,500 described species (Li *et al*., 2001).

Eggplant is a bushy plant and grows to a height of 60-150 cm. The plant is erect, compact and well branched. It has a rather fibrous or lignified root system. The leaves are large, simple, lobed, uneven leaf base and alternate on the stems. The flowers are large, violet or white colored, and solitary or in clusters of two or more. The stems, leaves, and calyx of some cultivars are spined. The fruit is a pendant, fleshy berry. The shape of fruit varies from ovoid, oblong, obovoid or long cylindrical. The color of fruit varies from (shiny) purple, white, green, yellowish, to striped. The seeds are borne on the fleshy placentae filling the locular cavity completely.

Eggplant is a warm weather plant that grows best at a temperature of 21°-29°C. It cannot tolerate frost, and the growth of young plants will be retarded when night temperature is below 16°C. Cool temperature and cloudiness can reduce fruit set. Eggplant can tolerate drought and excessive rainfall, but struggles to grow when temperature exceed 30°C. When temperature and humidity are high, eggplant becomes more vegetative. Eggplant prefers a soil that is deep fertile, well drained and high in organic matter and has a pH of 5.5- 6.8. A long growing season of about 120 days is required for successful eggplant production.

Eggplant is subjected to many diseases and pests which cause damage in all growth stages. Among the diseases of eggplant, bacterial wilt is one of the most devastating diseases, which severely hampers its cultivation. It is one of the most serious diseases occurring in tropics, subtropics and warm temperate regions of the world.

Ralstonia solanacearum is the causal pathogen of bacterial wilt, and was previously known as *Pseudomonas solanacearum* (Smith) (Yabuchi et al.1995). It has a wide host range. It has been classified into five races based on their host range. Race 1 has the broadest host range, which includes Solanaceous crops and legumes endemic to the southern United States. Race 2 of *R. solanacearum* is pathogenic to triploid banana and is mostly seen in Central America and Southeast Asia. Race 3 is restricted to potato and tomato and is distributed worldwide. Race 1 of biovar 3 *R. solanacearum* is pathogenic to eggplant.

This organism is a gram negative, non-spore forming and rod-shaped aerobic bacterium. Optimum growth occurs at a temperature range of 30-35°C and it does not grow above 41°C. In culture, colonies are cream coloured and slimy. When tissue from the base of infected stems is placed in water, ooze exudes. This pathogen has the ability to survive in soil and travel along water and it can associate with weeds without causing symptoms.

The bacterium generally invades plant roots via wounds or the natural openings from which secondary roots subsequently emerge (Hayward *et al*., 1991). After invasion, bacteria first colonize and proliferate in intercellular spaces and then invade xylem vessels. Once bacteria invade the vessels they multiply and travel rapidly throughout the entire plant. Sap flow is reduced by the presence of large numbers of bacteria and the exo-polysaccharide (EPS) slime that they produce when plants wilt (Schell *et al*., 2000). In the initial stage of disease, brownish

discolouration of vascular tissues occurs, which may be accompanied by browning and rotting of the tissue inside the vascular bundles (Smith, 1920). The disease is characterized by a sudden wilting of the plants at the flowering stage, yellowing of foliage and stunting of the plant growth (Kelman, 1953 and Rai *et al.*, 1975).

Because of the soil-borne nature of the pathogen, conventional management strategies like crop rotation, adjusting the date of planting, cultural methods and soil treatment are not effective, especially for its broad host range. The most successful strategy is to breed resistant cultivars or graft plants using resistant rootstocks. Bacterial wilt also can be controlled by the application of fertilizers and cost-effective soil amendments, such as addition of compost, manure, urea, calcium oxide or soil solarization, to change soil pH and reduce survival and activity of plant pathogens (Gorissen et al., 2004). The wilt caused by *Ralstonia solanecearum*, causes yield loss ranging from 4.24 to 86.14% (Sabita *et al*., 2000).

Until recently, very few resistant eggplant cultivars have been developed, due to the lack of resistant resources. Resistance sources have been identified in some wild relative species, such as *Solanum torvum* and *Solanum aethiopicum* (Gousset *et al*., 2005). The resistance is greatly affected by environmental factors, race and strain diversity of the pathogen, makes it very difficult to utilize these resistance sources in different countries.

Since the last five decades, molecular markers were playing a greater role in genetic improvement, disease resistance, germplasm characterization, mapping, gene tagging, QTL analysis, paternity analysis, finger printing and utilization in a wide range of horticultural crops. Molecular markers tightly linked to resistance genes can obviate the need for phenotype testing to identify resistant individuals in

resistance-segregating breeding populations through Marker-Assisted Selection (MAS). Markers such as RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), SSR (Simple Sequence Repeats) and SNPs (Single Nucleotide Polymorphisms) have been found very useful tools (Agarwal *et a*l., 2008).

At present, very few eggplant varieties/hybrids from public and private sector showing resistance against bacterial wilt was breed. Since *Ralstonia solanecearum* is highly unstable in expression, breeder requires a molecular tool to introgress the resistance gene to high yielding varieties. The following study was taken up with the following objectives:

1. Phenotyping of brinjal genotypes (IIHR-500A, IIHR-575 and F_2 population) for bacterial wilt resistance.
2. Identification of polymorphic SSR markers for the parents (IIHR-500A and IIHR-575) used in the mapping population.
3. Identification of polymorphic SSR markers linked to bacterial wilt in brinjal in F_2 population.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

In this chapter the literature pertaining to various aspects of the study are reviewed in brief under the following heads:

2.1.Morphological and biochemical features of *Ralstonia solanacearum*

2.2. Genetics and resistance breeding for Bacterial Wilt

2.3. Molecular markers

2.4. Marker assisted selection

2.5. DNA based markers

2.6. Bulk Segregant Analysis (BSA)

Brinjal or eggplant (*Solanum melongena* L.) is one of the important solanaceous vegetable crops grown worldwide. It is a hardy crop and easily grown throughout the world and year round in tropical and sub-tropical regions except in higher altitudes where it succumbs to frost injury. Brinjal production has been hampered because of bacterial wilt caused by *Ralstonia solanacearum* (Yabuchi *et al.*, 1995). During recent years, various varieties, modern technologies and voluminous literature have been reported in this crop. Still, identification of resistance source for the development of resistant hybrid/variety with good resistance is a basic need.

2.1.1 Morphological and biochemical features of *Ralstonia solanacearum*

Ralstonia solanacearum (Smith) (Yabuchi *et al.* 1995) (Formerly called *Pseudomonas solanacearum*), is a soil-borne bacterial pathogen and a major limiting factor in the production of many crop plants around the world. This organism is the causal agent of brown rot of potato,

bacterial wilt or southern wilt of tomato, tobacco, eggplant, some ornamentals and Moko disease of banana (Agrios, 1997).

Ralstonia solanacearum is widely distributed and found in tropical, subtropical and in some temperate regions of the world. The species as a whole has a very broad host range and infects hundreds of species in many plant families. The majority of hosts are dicots with the major exception being bananas and plantains. Most economically important host plants are found in the Solanaceae or nightshade family (Stevenson *et al.*, 2001). *Ralstonia solanacearum* is a gram-negative motile rod. The organism grows aerobically and does not form endospores. Cells are (0.5-0.7 X 1.5-2.0) µm and are non-encapsulated. *Ralstonia solanacearum* is catalase positive, oxidase positive and reduces nitrates. The pathogen does not hydrolyze starch and does not readily degrade gelatine.

Specific host range and distribution of *R. solanacearum* depends on the race and to some degree the biovar of the pathogen. *R. solanacearum* is a highly variable species encompassing six biovars based on biochemical properties (Hayward *et al.*, 1991; He *et al.*, 1983) and five races based on host range (Buddenhagen *et al.*, 1962, Pegg *et al.*, 1971). These host ranges and distributions are changing in recent years (Denny, 2000).

For a quick field diagnostic identification of *Ralstonia solanacearum* and to distinguish bacterial wilt from vascular wilts caused by fungal pathogens, bacterial streaming from infected plant material can be used. A stem section is cut from a plant with vascular discoloration using a sharp knife or razor blade. The stem section is placed against the inside wall of a water-filled clear beaker or test tube so that the end of the section just touches the water surface. Milky white strands containing

bacteria and extracellular polysaccharide will stream from the cut ends of the xylem (Shew *et al.*, 1991).

Molecular detection methods are considered to be highly specific, sensitive and rapid. Strains of *Ralstonia solanacearum* isolated from bacterial wilt affected plants of brinjal, chilli and tomato were compared based on the utilization of carbohydrates, hypersensitivity reaction on capsicum leaves and RAPD analysis and based on the results the strains are grouped into various biovars and races (James *et al.,* 2003).

2.1.2. Epidemiology of *Ralstonia solanacearum*

The pathogen infects roots through wounds and colonises the vascular tissue causing plugging of the xylem and leaf wilting. There is often an association between nematode infection and bacterial wilt, where the nematodes create wounds in the root tissue to allow an entry point for the bacterium to infect the plant.

The bacterium survives in the soil and can maintain infectious populations over several years.

Incubation period of the bacterium is longer in resistant and moderately susceptible accessions (Hussain *et al.*, 2005).

Alternative weed hosts may also play a role in survival and overseasoning.

Temperature is a major determinant in the distribution of this pathogen, which is widespread in tropical, sub-tropical and warm temperate regions where the mean soil temperature is greater than 15°C.

Wet soil increases the incidence of disease and water movement contributes to the dissemination of inoculum.

The incidence and rate of wilting therefore increases with high temperatures and soil moisture.

Continuous cropping of susceptible plants will also favour infection.

The pathogen is disseminated by:

- Contaminated farming equipment, soil on tyres and footwear.
- Drainage water carrying inoculum through the soil.
- Infected seed, especially in the case of groundnut.
- Seedlings raised in infected soil, spreading the pathogen to new areas.
- Cultural operations such as pruning.

2.2. Genetics and resistance breeding for Bacterial Wilt.

2.2.1. Genetics of Bacterial Wilt

The study of inheritance pattern of resistance to biotic stresses is essential for developing resistant hybrid/variety. The bacterial wilt inheritance is race, biovar, strain, genotype and environment specific and it is genetically determined. According to Akiba (1972), the resistance to bacterial wilt in '*Nihan nassu'* cultivar was due to the presence of single dominant gene. Gopinath and Madalageri (1986) reported that resistance to bacterial wilt was inherited as single dominant gene character in the cross WCGR-112-8 and Pusa kranti in brinjal.

Feng *et al.* (2003) concluded that the inheritance of bacterial wilt resistance as additive-dominant model. Additive, dominant and recessive effects played major role in the genetics of bacterial wilt resistance, with

additive effects being the dominant factor. Zhu *et al.*, 2004, revealed that the resistance of bacterial wilt was controlled by single dominant gene.

Gopalakrishnan *et al.* (2005) demonstrated, inheritance study of susceptibility to bacterial wilt was dominant over resistance in brinjal. Ajjappalavara *et al.,* 2008, showed that the segregation of population to the inheritance of bacterial wilt resistance was 3 (non-wilted):1 (wilted) ratio and suggested single gene inheritance for bacterial wilt resistance.

2.2.2. Resistance breeding for bacterial wilt

Since agro-chemicals are not effective and sanitary cropping systems difficult to apply (Hanudin *et al.*, 1992), control strategies of disease resistance have so far mainly concentrated in plant breeding. Although screenings of eggplant accessions were conducted to find sources of resistance and despite the fact that some resistant varieties have been developed (Rao *et al.*, 1976, Goth *et al.,* 1991, Peter *et al.*, 1993,), the level of resistance has become insufficient in hot planting season or poorly drained fields (Ano *et al.*, 1991). Traits of resistance against bacterial wilt have been identified in different wild relatives of eggplant, such as *Solanum torvum*, *S. sisymbrifolium* (Dauny *et al.,* 1991).

Solanum aethiopicum is reported to carry resistance to bacterial wilt disease caused by *Ralstonia solanacearum*, which is one of the most important diseases of eggplant (*Solanum melongena* L.). Somatic hybrids between *Solanum melongena* cv. Dourga and two groups of *Solanum aethiopicum* produced by electrical fusion of mesophyll protoplasts were found tolerant to *Ralstonia solanacearum*, as about 50% of plants wilted. All somatic hybrids tested were as tolerant as the wild species (Collonnier *et al.*, 2001).

Rizza *et al.* (2002) showed that dihaploid plants obtained through anther culture of somatic hybrids between eggplant and *Solanum aethiopicum* and *Solanum gilo* showed complete resistance to fungal wilt caused by *Fusarium oxysporum* f. sp. *melongenae*. The population of androgenic plants developed may represent a useful source for introgression of the *Fusarium* resistance trait into eggplant.

Solanum torvum Sw., a wild species closely related to eggplant (*Solanum melongena* L.), has been identified as a potential source of resistance to bacterial wilt disease for cultivated susceptible *Solanaceae* species. It has been found that *Solanum torvum* can be considered as resistant to the race 1 biovar 1 strain, and tolerant to the race 1 biovar 3 and race 3 biovar 2 strains of *Ralstonia solanacearum* (Clain *et al.*, 2004).

Accessions of *Solanum torvum* Sw., assessed for morphology, fertility, levels of resistance against *Ralstonia solanacearum* showed bacterial wilt symptoms on lower leaves of *Solanum torvum* plants tested without causing any plant death. The presence of bacteria serologically detected in roots of symptomless plants, suggested *Solanum torvum* to be tolerant to *Ralstonia solanacearum* (Gousset *et al.*, 2005).

Solanum commersonii, a wild tuber-bearing species native to Uruguay with high potential for use in potato breeding programs has been found to carry resistance against the bacterial pathogen *Ralstonia solanacearum*, the causative agent of bacterial wilt (Laferriere *et al*., 1999). Leaf extracts of several Uruguayan *Solanum commersonii* accessions collected in different geographic locations were shown to produce an inhibitory effect on the growth of *Ralstonia solanacearum* suggesting the presence of constitutive compounds associated with resistance (Siri *et al*., 2005).

Fertile somatic hybrids between *Solanum integrifolium* and *Solanum sanitwongsei* (syn. *Solanum kurzii)* have been used as candidates for bacterial wilt-resistant rootstock of eggplant (Iwamoto *et al.*, 2007).

Toppino *et al*. (2008) studied the inheritance of resistance introgressed from *Solanum aethiopicum Gilo* and *Aculeatum* groups into cultivated eggplant (*Solanum melongena* L.) to develop and validate the associated PCR-based molecular markers associated with fusarium wilt disease of eggplant.

2.3. Molecular Markers

Traditional plant breeding has gone through many phases, from the era of cross pollination between varieties of the same species, to hybridisation between different species and genus to overcome barriers imposed by combining species belonging to different families. However, traditional plant breeding is expensive and time consuming and moreover, the selection and evaluation of the new varieties can take several years to achieve. With the discovery of DNA, a new area of modern plant biotechnology began. In plant breeding the development of molecular marker systems facilitated the selection and evaluation process greatly. These molecular tools have increased the speed and precision for achieving desired agronomic traits.

The development of molecular techniques for genetic analysis has led to a great augmentation in knowledge of crop genetics and understanding of the structure and behaviour of various crop genomes. These molecular techniques, in particular the applications of molecular markers, have been used to scrutinize DNA sequence variation(s) in and among the crop species and create new sources of genetic variation by introducing new favourable traits.

Markers can aid selection for target alleles that are not easily assayed in individual plants, minimize linkage drag around the target gene, and reduce the number of generations required to recover a very high percentage of the recurrent parent genetic background. Improvements in marker detection systems and in the techniques used to identify markers linked to useful traits, has enabled great advances to be made in recent years.

A genetic marker is a DNA sequence with a known location on a chromosome and associated with a particular gene or trait. It can be described as a variation, which may arise due to mutation or alteration in the genomic loci that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, SNP), or a long one, like minisatellites. A molecular marker is defined as a particular segment of DNA that is representative of the differences at the genome level. Molecular markers may or may not correlate with phenotypic expression of a trait. Thus offers numerous advantages over conventional phenotype based alternatives as they are stable and detectable in all tissues regardless of growth, differentiation, development or defence status of the cell are not confounded by the environment, pleiotropic and epistatic effects.

An ideal molecular marker technique should have the following criteria: (1) be polymorphic and evenly distributed throughout the genome; (2) provide adequate resolution of genetic differences; (3) generate multiple, independent and reliable markers; (4) simple, quick and inexpensive; (5) need small amount of tissue and DNA samples; (6) have linkage to distinct phenotypes and (7) require no prior information about the genome of an organism (Aggarwal *et al.*, 2008).

2.4. Marker Assisted Selection

Marker-assisted selection is the process of selection of plants carrying desired genes with the use of molecular markers. With the development and availability of an array of molecular markers and dense molecular genetic maps in crop plants, MAS has become possible for traits both governed by major genes as well as quantitative trait loci (QTLs).

DNA markers that are tightly linked to agronomically important genes (called gene 'tagging') may be used as molecular tools for marker-assisted selection (MAS) in plant breeding (Ribaut and Hoisington, 1998). MAS exploits the presence or absence of a marker as a substitute for or to assist in phenotypic selection, in a way which may make it more efficient, effective, reliable and cost-effective compared to the more conventional plant breeding methodology. The use of DNA markers in plant breeding has opened a new realm in agriculture called 'molecular breeding' (Rafalski and Tingey, 1993).

2.5. DNA based markers

DNA based assays have revolutionised and modernised our ability to characterise genetic variation. The first advantage of molecular techniques is their capacity to detect genetic diversity at a higher level of resolution; furthermore DNA based assays are robust, speedy, information may be obtained from the little amounts of plant material at any stage of development and it is not affected by environmental conditions. A large number of different molecular techniques are at present available and each of them differs in its informational content.

The term 'marker' implies that the loci detected are anonymous and phenotypically neutral. These DNA based makers differentiate the

organisms at DNA level and are inherited in simple Mendelian fashion (Waltson, 1993). They are particularly important for genetic resource management and for the rational use of genetic resources in selection programs. An ideal genetic marker should be polymorphic, multi-allelic, co dominant, non-epistatic and it should be insensitive to environment (Winter and Kahl, 1995).

Molecular markers are of great importance by being the main source for many genetic analyses and for crop improvement programmes. DNA / molecular markers are employed in two broad areas in plant breeding (i) molecular profiling or genome analysis to facilitate the identification of genotypes or assessment of genetic distance and diversity and (ii) Marker assisted selection (MAS) in plant breeding. Different classes of markers are employed to attain the above-mentioned goals and these include RFLP, AFLP, RAPD, SRAP, SCAR, SSR (Microsatellite).

2.5.1. Restriction Fragment Length Polymorphism (RFLP) markers

Restriction Fragment Length Polymorphism is the original DNA marker, and was developed in the late 1970's (Botstein *et al.*, 1980). Development of this technique was facilitated by the discovery of restriction enzymes. It is a co-dominant marker where DNA is digested with restriction enzymes; the fragments are separated based on size using agarose gel electrophoresis. Then transferred in denatured form to a nylon membrane, which is then fragments are incubated in a solution containing a probe previously labelled, either radioactively or chemically for hybridisation. RFLPs were first used by Grodzicker *et al.* (1974) in retrovirus and later found its applications in many crop improvement programmes.

However, RFLP analysis is expensive, laborious, time consuming involves the use of hazardous radioactive isotopes and need large amount of good quality DNA. Isshiki *et al.*, 2003, performed RFLP analysis of mtDNA for assessing phylogenetic relationships in eggplant and related *Solanum* species at the interspecific level.

2.5.2. Random Amplified Polymorphic DNA (RAPD) markers

Its simplicity and low cost involvement has made it very handy and widely used techniques by geneticists. It is now being used extensively in place of RFLP (Welsh and Mc Cleland, 1994; Williams *et al.*, 1990). This detects polymorphism by a single primer of arbitrary nucleotide sequence using PCR. The amplified products are separated by agarose gel electrophoresis. The major advantage of this assay over RFLP is that, there is no need for prior information on the genomic DNA sequences, universal set of primers can be used for a variety of species, and the process is automated.

Nunome *et al.* (2001) scored the fruit shape and color development trait and investigated the linkage to RAPD and AFLP markers. The fruit shape showed a significant association with markers on linkage group 2. Color development in fruit, stem and calyx showed a significant association with markers on linkage group 7.

Bacterial wilt caused by *Burkholderia caryophylli* is one of the most important and damaging diseases of carnations (*Dianthus caryophyllus*) in Japan. The RAPD marker WG44-1050 was successfully converted to a sequence-tagged site (STS) marker suitable for marker-assisted selection (MAS). In addition, the STS marker developed was useful and reliable as a selection marker for breeding for resistance to bacterial wilt, using a highly resistant wild species, *D. capitatus* ssp. *andrzejowskianus* and a

resistant line, 'Carnation Nou No. 1', as breeding materials (Onozaki *et al.*, 2009).

2.5.3. Sequence Related amplified polymorphism (SRAP)

The SRAP technique (Li and Quiros, 2001) consists of preferential amplification of ORFs using PCR. For this purpose, combinations of two types of primers were employed. The first type of primer (forward) is 17 bp long, and contains a fixed sequence of 14 nucleotides rich in C and G, and three selective bases at the 3' end. This primer preferentially amplifies exonic regions, which tend to be rich in these nucleotides. The second type of primer (reverse), with 18 bp, contains a sequence of 15 nucleotides, rich in A and T, and three selective bases at the 3' end. This primer preferentially amplifies intronic regions and regions with promoters, rich in these nucleotides.

Jian and Jun (2006) used bulked segregant analysis to identify SRAP markers linked to bacterial wilt resistance in the two segregating populations of diploid potato. A SRAP marker M32 linked to resistant gene RBW was screened from 88 pairs of combination with 8 forward primers and 11 reverse primers. The genetic distance between the M32 and site of bacterial wilt resistance was 10.2 cM and 17.3 cM in ED and CE populations respectively. Chen *et al.,* 2010 employed sequence-related amplified polymorphism (SRAP) markers to evaluate genetic variation in a diverse collection of 56 *Solanum* accessions.

Mutlu *et al.* (2008) reported the tagging of the gene for resistance to fusarium wilt (FOM) in eggplant using SRAP, RGA, SRAP-RGA and RAPD markers and confirmed the monogenic inheritance of resistance. These markers provide a starting point for mapping the eggplant FOM resistance gene in eggplant and for exploring the synteny between solanaceous crops for fusarium wilt resistance genes.

2.5.4. Amplified fragment length polymorphism (AFLP) marker

Amplified fragment length polymorphism involves digestion of DNA with the restriction enzymes as in case of RFLP and PCR amplification. AFLP combines the reliability of RFLP with the power of PCR technique. PCR amplification of restriction fragments is carried out using oligonucleotide adapters (Vos *et al.*, 1995). Fingerprints can be produced without prior knowledge using limited sets of generic primers. This method generates large number of DNA fragments facilitating detection of polymorphism. AFLPs are also dominant and can detect large number of polymorphisms.

Poussier *et al.* (2000) used AFLP markers to assess the genetic diversity among strains in a worldwide collection of *Ralstonia solanacearum*, causal agent of bacterial wilt, which permitted very fine discrimination between different isolates.

A cross between bacterial wilt resistant tomato variety “T51A”and susceptible variety “T9230” was made for mapping bacterial wilt resistance gene. AFLP analysis was performed on two parents and their F_2 resistant and susceptible bulks and the DNA fragment thought to be closely linked to one of the bacterial wilt resistant genes was found (Shou *et al.*, 2006)

Tomato genotypes both susceptible (H24) and resistant (Anagha) to Bacterial wilt were used to monitor the expression of stress induced genes at the transcript level. cDNA-AFLP (Amplified Fragment Length Polymorphism) analysis was performed with the cDNA synthesized from resistant and susceptible genotypes at second and fifth days after inoculation. 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 present study

would be helpful in elucidating the molecular basis of the infection process and identifying the defense genes that can be targeted for incorporating bacterial wilt resistance (Nazeem *et al*., 2011)

2.5.5. Sequence characterized amplified region (SCAR)

Sequence characterized amplifiedregion (SCAR) markers are more reproducible and easier to manipulate in marker-assisted selection (MAS) programs than other markers. Due to the co-dominant or dominant nature, SCAR marker can provide a valid tool for the accurate assessment of genotype at the linked locus. Furthermore, it is useful to map whole F_2 populations without losing genetic information and it can discriminate between different alleles identifying homozygous and heterozygous plants in segregating populations. As a result, SCAR can be considered to be an ideal marker for plant breeding programmes.

Bi-hao *et al.* (2009) showed that the inheritance of bacterial wilt resistance in eggplant was controlled by a single dominant gene showing Mendelian inheritance model. In addition, a 762 bp molecular marker linked to a bacterial wilt-resistant gene of eggplant was screened by the bulked segregant analysis (BSA) method and sequence characterized amplified region (SCAR) marker linked to bacterial wilt-resistance gene was also obtained.

2.5.6. Simple sequence repeats (SSR) markers

Microsatellites are tandem repeats of DNA sequences of only a few base pairs (1-6 base pairs) in length, the most abundant being the dinucleotide repeats. These are most important class of markers which are abundant with long hyper variable region. $(CA)_n$ repeat is one of the most frequently occurring microsatellite in human and many mammalian genomes. In contrast (AT) n is more abundant in plants. Variation in the

number of tandemly repeated core nucleotide sequences at SSR locus among different genotypes provides the basis for polymorphism that can be used in genetic studies. Their value for genetic analysis lies in their multi-allelism, co-dominant inheritance, relative abundance, genome coverage and suitability for high-throughput PCR-based platforms (Tumbilen *et al*., 2011). The DNA sequences flanking SSRs are conserved (Pervaiz *et al.,* 2010) and these conserved sequences have been used in designing suitable primers. Co-dominance of alleles, high genomic abundance in eukaryotes and random distribution throughout the genome, with preferential association in low copy region (Morgante *et al.,* 2002) and low quantities of template DNA required are characteristic features of SSR markers.

High development cost is a major impediment to the routine application of SSRs in the genetic studies of non-commercial species and for identifying markers located in chromosomal regions of interest. The development of locus-specific SSR markers requires the isolation and characterization of individual loci, a process involving the construction and screening of a DNA library with microsatellite-specific probes, followed by DNA sequencing of positive clones and subsequent PCR primer synthesis and testing. The recovery rate of useful SSRs is generally low due to non-specific amplification and monomorphic loci. In addition, the random selection of clones prevents predetermination of the chromosomal location and copy number of microsatellite loci.

As the flanking DNA is more likely to be conserved, the micro-satellite-derived primers can often be used with many varieties and even other species. These markers are easily automated, highly polymorphic, and have good analytical resolution, thus making them a preferred choice of markers (Gupta *et al*., 1994).

SSRs tend to cluster at the centromeres and telomeres. However, this problem can be solved by developing SSRs from EST libraries, which are gene rich and more evenly distributed. It was long assumed that SSRs were primarily associated with non-coding DNA, but it has now become clear that they are also abundant in the single and low-copy fraction of the genome. These latter SSRs are commonly referred to as "genic SSRs" or "EST-SSRs" and are present in 1 to 5% of the expressed gene. Genomic SSRs are reported to be collected around particular regions of the chromosomes, such as centromeric areas and the plant DNA sequences deposited in public databases. *S.melongena* having genotype of Angio5 long green is mostly used for development of breeding lines (Stagel *et al*., 2008). A large number of SSR based techniques have been developed and a quantum of literature has accumulated regarding the applicability of SSRs in plant genetics and genomics (Kalia *et al.,* 2010).

Twenty-seven tomato materials collected from four countries were evaluated in bacterial wilt resistance with artificial inoculation and PCR analysis were performed with SSR markers to identify the potential marker linked to bacterial wilt resistance. The results showed that "Xiangyin79-1"was highly resistant,"860" and "039-3"were resistant to this disease. An SSR marker Tom176-177 had a band pattern different from all other materials with less resistance which was inferred to be linked with bacterial wilt resistance (Lin *et al.*, 2007).

Wang *et al.* (2008) used SSR markers to reveal genetic diversity in eggplant. Eighty eight accessions including eighty six of *Solanum melongena* and two wild related species (*Solanum integrifolium* and *Solanum torvum*) were used to analyze the genetic variation with 23 SSR primers.

Nunome *et al.* (2009) constructed simple sequence repeat (SSR)-enriched genomic libraries in order to develop SSR markers, and sequenced more than 14,000 clones. cDNA libraries were constructed from several eggplant tissues and 6,144 expressed sequence tag (EST) sequences were obtained. From these sequences, 209 primer pairs were designed, 7 of which segregated in the mapping population. On the basis of the segregation data a linkage map was constructed, and the 236 segregating markers were mapped to 14 linkage groups.

Sunseri, *et al.* (2010) assessed the genetic diversity and evaluated genetic relationships among 70 “scarlet eggplant” (*Solanum aethiopicum* L.) using Simple Sequence Repeat (SSR) analyses. The clusters obtained through cluster analysis did not show any relationships with geographic origins and/or botanical groups. Matrices of genetic similarity from SSR data were utilized in order to obtain a dendrogram.

More recently, *in silico* mining of microsatellite sequences from DNA-sequence databases has been an alternative for obtaining microsatellite markers (SSRs) from genomic libraries (Stàgel *et al.*, 2008).

An integrated DNA marker linkage map of eggplant (*Solanum melongena L*.) was constructed which consisted of 12 linkage groups and encompassed 1,285.5 cM in total. Mapping 952 DNA markers, including 313 genomic SSR markers developed by random sequencing of simple sequence repeat (SSR)-enriched genomic libraries, and 623 single-nucleotide polymorphisms (SNP) and insertion/deletion polymorphisms (InDels) found in eggplant- expressed sequence tags (ESTs) and related genomic sequences[introns and untranslated regions (UTRs)]. Because of their co-dominant inheritance and their highly polymorphic and multi-allelic nature, the SSR markers may be more versatile than the SNP and InDel markers for map-based genetic analysis of any traits of interest

using segregating populations derived from any intraspecific crosses of practical breeding material (Fukuoka *et al.,* 2012).

2.5.7. EST-SSR markers

Currently plant biologists are exploiting the use of Expressed Sequence Tags (ESTs) markers in gene discovery research. ESTs are short DNA sequences corresponding to a fragment of a complimentary DNA (cDNA) molecule and which may be expressed in a cell at a particular given time (Gupta *et al*., 2003). One of the many interesting applications of ESTs database (dbEST) is gene discovery where many new genes can be found by querying the dbEST with a protein or DNA sequence. ESTs are relatively conserved region of the genome. ESTs thus represent informative source of expressed genes and provide a sequence resource that can be exploited for large-scale gene discovery (Boguski *et al.,* 1993).

Locus-specific primers flanking EST- or genic SSRs can be designed to amplify the microsatellite loci present in the genes. Thus, the generation of (genic) SSR markers is relatively easy and inexpensive because they are a by-product of the sequence data from genes or ESTs that are publicly available.

Stàgel *et al.* (2008) used a set of eggplant EST-SSR markers for phylogenetic analysis and genetic mapping. Since EST-SSRs lie within expressed sequence, they have the potential to serve as perfect markers for genes determining variation in phenotype.

2.6. Bulk Segregant Analysis (BSA)

Bulk segregant analysis is a method for rapidly identifying markers linked to any specific gene or genomic region. Bulk segregant analysis involves screening for differences between two pooled DNA samples derived from a segregating population that are originated from

a single cross. Two pools contrasting for a trait (e.g., resistant and susceptible to a particular disease) are analyzed to identify markers that distinguish them. Therefore, the two resultant bulked DNA samples differ genetically only in the selected region and are seemingly heterozygous and monomorphic for all other regions. Markers that are polymorphic between the pools will be genetically linked to the loci determining the trait used to construct the pools. Linkage between a polymorphic marker and the target locus is confirmed and quantified by using the segregating population from which the bulks were generated. Probes or primers for loci that are polymorphic and absolutely linked to the gene or region used to distinguish the individuals comprising the bulks will detect clear differences between the bulks. In contrast, unlinked loci will appear heterozygous with approximately equal band intensities in each bulk. Recombination between the target marker and the assayed polymorphic locus will result in diminishing distinction between the two bulks with decreasing linkage until the locus appears unlinked. Bulk segregant analysis does not reveal novel types of variation but rather allows the rapid screening of many loci and therefore the identification of segregating markers in the target region (Michelmore *et al.*, 1991).

The bulked segregant analysis (BSA) was used on *Larix leptolepis* × *L. olgensis* hybrids to identify a RAPD marker associated with high rooting ability in larch. Two DNA bulks: H (high rooting ability) bulk and L (low rooting ability) bulk were constructed according to the rooting percentages of stock plants. Out of the 328 primers used, only S356 had amplified a specific band, named S356445, which only existed in the H bulk. This was further confirmed following selective genotyping of individual hybrids. The sequence, S356445 was converted to a SCAR marker ‘HRL445’ which was useful in MAS to screen for larch with high rooting ability (Li *et al.,* 2008).

BSA approach was used in sweet potato (*Ipomoea batatas* L.) to develop AFLP markers linked to the stem nematode resistance. The 800 AFLP primer combinations were screened in the resistant and susceptible bulked DNA from the 186 progeny of an F_1 single-cross population of Xu781 (resistant parent) x Xushu18 (susceptible parent) and 245 AFLP primers showed polymorphic bands between resistant and susceptible DNA. Primer combinations detecting polymorphism between the two bulks were used to screen the parents and eight individuals from each of the bulks. E2M23 and E33M20 produced a specific band of about 500 bp and 200 bp in length in resistant plants but not in susceptible plants. $E2M23_{500}$ and $E33M20_{200}$ were linked to a gene for stem nematode resistance. These two AFLP markers were used in marker-assisted breeding for stem nematode resistance in sweet potato (Qin *et al.,* 2009).

The bulked segregant analysis (BSA) was used for identifying SNP markers in rapeseed by deep sequencing a representative library and performing bulk segregant analysis. With this method, SNPs associated with rapeseed pod shatter-resistance were discovered. Firstly, a reduced representation of the rapeseed genome was used. Genomic fragments ranging from 450–550 bp were prepared from the susceptible bulk (ten F_2 plants with the silique shattering resistance index, SSRI, 0.10) and the resistance bulk (ten F_2 plants with SSRI .0.90), and also Solexa sequencing produced 90 bp reads. Approximately 50 million of these sequence reads were assembled into contigs to a depth of 20-fold coverage. The distribution of these SNPs appeared a tight cluster, which consisted of 14 associated SNPs within a 396 kb region on chromosome A09.Finally, two associated SNPs were mapped on a major QTL region (Hu *et al.,* 2012).

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The present investigation was conducted on brinjal plants at the Division of Biotechnology, IIHR. The details of preparation of experimental site, growing of plants, bacterial inoculum preparation, infecting the plants, isolation of DNA, detection of parental polymorphism, screening of brinjal genotypes for *Ralstonia* resistance, identification of markers for wilt resistance, the details of experiments, materials used and techniques adopted in the present investigation are presented in this chapter.

3.1. EXPERIMENT 1: Phenotyping of brinjal genotypes for bacterial wilt resistance

3.1.1. Experimental site

The disease screening for bacterial wilt resistance in brinjal was conducted in the polyhouse of Division of Biotechnology, Indian Institute of Horticultural Research (IIHR), Bangalore during December, 2011. The molecular aspects of screening of markers linked to bacterial wilt resistance in brinjal was carried out in the Molecular Biology Lab, Division of Biotechnology, IIHR, Bangalore.

Selection of varieties

The two parental genotypes that were selected for this study had the following morphological characters:

IIHR-500A: Plants are medium tall with dark green stem and dark green leaves. Flowers purple with fleshy green calyx. Fruits are oblong in shape, light purple colour with white stripes. Plants are highly resistant to bacterial wilt. (Plate 2)

Plate 1: Representation of parental plant population.

(a) **(b)**

Plate 2: (a) Individual plants 0f IIHR-500A with the fruit type (Parent 1, Resistant to bacterial wilt); (b) IIHR-575 with the fruit type (Parent 2, susceptible to bacterial wilt) with fruit respectively.

IIHR-575: Plants are tall and of bushy; stem and leaves are green in colour. Stem is less hard with a feature of thorniness. Flowers are purple in colour with fleshy green calyx. Fruits are oval with flat base. Fruits are purple in colour with white stripe. Plants are highly susceptible to bacterial wilt.(Plate 2)

Details of Experiment

Brinjal seeds were sown in trays containing coco pith and allowed to germinate. After 21 days of sowing or at 3 leaf stage, the young seedlings were transplanted to pot filled with soil, well-rotten FYM and sick soil from wilt infested plots (10:2:4 ratio).

3.1.2. Bacterial inoculum preparation

Collection of *Ralstonia* infected samples

Ralstonia solanacearum infected brinjal plants were collected from *Ralstonia* infested sick plot. The presence of pathogen was tested by placing longitudinal sections containing vascular tissue from diseased plants in a test tube with distilled water. The infected tissue showed fine streaks of milky ooze and composed of masses of bacteria which come out from the margin of the cut portion within a few minutes. This distinguishes bacterial wilt from other wilts like fusarium wilt and physiological wilt. The ooze-out was plated on Triphenyl Tetrazolium Chloride (TTC) medium.

Isolation of *Ralstonia* using TTC media

Bacterial inoculum was prepared from the virulent colonies (irregular shaped, white mucoid with pink colour at centre) grown on Triphenyl Tetrazolium Chloride (TTC) medium. The composition of Triphenyl Tetrazolium Chloride (TTC) medium is given in (table 1) as follows:

Table 1. Composition of Triphenyl Tetrazolium Chloride medium (for litre) at PH : 7

Sl No.	Composition	Quantity
1	Dextrose	10g
2	Peptone	10g
3	Casein	1g
4	Agar Agar	20g
5	Distilled Water	1lit

To the autoclaved medium, 5 ml of filter sterilized 1% TTC was added for 1 litre of media was added maintaining a final concentration of 50 mg/ml. Bacterial colonies were seen after 48 hours of incubation at a temperature of 30˚C.

Preparation of inoculum

The pure culture of the bacterium was cultured on Nutrient broth (NB) (Table 2)

Table 2. Composition of nutrient broth (for litre) at PH: 7

Sl No.	Composition	Quantity
1	Peptone	5g
2	Nacl	5g
3	Beef extract	3g
4	Distilled Water	1lit

After autoclaving of NB at 121˚C under 15lbs pressure for 30 minutes, the broth was kept for cooling. 7ml of bacterial suspension was added to the broth and incubated on rotary shaker for 48 hours at 30˚C.

The bacterial inoculum (Plate 3) was prepared and concentration of the suspension was adjusted to 0.3 O.D. at 600nm (approximately 10^8 cfu\ml) using spectrophotometer.

3.1.3. Plant root inoculation

25 day old plants were used for bacterial inoculation. Root incision method was used for infecting the plant. Incisions were made on roots of plants by making a sharp cut with knife. 5ml of bacterial suspension of 0.3 O.D. at 600nm per plant was injected using a syringe into the incisions (Plate 4)

3.2. EXPERIMENT 2: Identification of polymorphic markers for parents (IIHR-500A and IIHR-575) used in mapping population

3.2.1. Optimization of protocol for DNA isolation and purification

Sample preparation

Third to fourth young, healthy leaf from the brinjal plants before transplanting were collected using sterile blade/ scissors and brought to wet lab immediately and proceeded with the DNA extraction protocol. The leaves were washed thoroughly with distilled water and air dried to remove external moisture.

3.2.1.1. Isolation of genomic DNA by CTAB using “mini-prep” method

1. Preheating of 1ml extraction buffer with 5 l of 0.5% -mercapto ethanol to 60 C was done by keeping in water bath.
2. 100mg of leaf tissue was ground to fine powder using liquid nitrogen. A pinch of PVPP was added and mixed. The contents were transferred into 2ml capacity eppendorf tube containing 1ml CTAB buffer pre-heated to 60 C and shaken gently.

PLATE 3: Nutrient broth with *Ralstonia* culture growth

(a) **(b)** **(c)**

PLATE 4: Infection of plants with *Ralstonia* culture, (a) Incision to the plant root, (b) Incisioned root for infecting plants with *Ralstonia* culture, (c) Inoculation of *Ralstonia* culture to the incisioned root.

PLATE 5: Bacterial ooze emerging from root of diseased plant into water

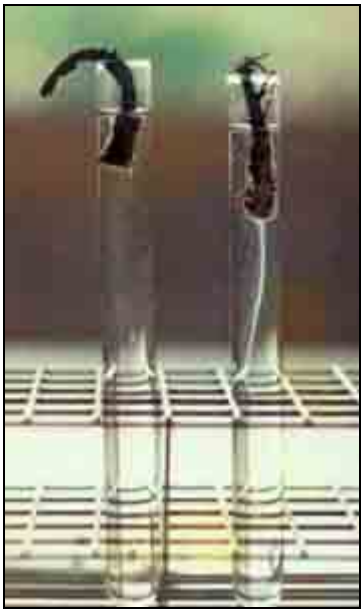

PLATE 6: Comparison between plants producing ooze and not producing ooze (Ref: www.lane-ag.org)

3. The tubes were incubated for 1hr at 60 C on water bath, shaken intermittently every 10 minutes and later cooled to room temperature.
4. 1ml of chloroform: Iso-amyl alcohol (24:1) solution was added and mixed gently by inverting tubes about 25 times to form an emulsion.
5. Centrifugation was done at 5000 rpm for 10 minutes and the aqueous phase was transferred to new eppendorf tubes using cut tips.
6. 50µl of 5M NaCl followed by 1ml of ice cold absolute ethanol was added and mixed gently. It was then refrigerated overnight at –20 C.
7. Centrifugation was done at 12000 rpm for 15 mins at 4˚C. The supernatant was poured off and the pellet was washed with 1ml 76% ethanol and centrifuged at 12000 rpm for 10 mins.
8. The washing step was repeated once more.
9. The supernatant was drained out to remove ethanol without completely drying DNA, by leaving tubes uncovered at 37 C for 20-30 min or vacuum drying at room temperature.
10. The pellet was re-suspended in 200µl TE buffer.
11. RNAase was added to a final concentration of 10 g/ml and incubated at 37 C for 30 min for immediate analysis or stored at (–) 20 C for future use.

3.2.1.2. Purification of DNA

1. The samples were diluted in 200µl of TE buffer. 50µl of 7.5M Ammonium acetate was added followed by 700 µl of cold absolute

ethanol and gentle mixing was done to precipitate DNA and incubated for 1 hour at (-)20˚C.

2. Finally tubes were centrifuged at 12000 rpm for 20 minute at 4˚C and the supernatant was decanted.
3. The DNA pellet was dried at 37˚C for 15 minute and dissolved in 100µl of TE buffer.

3.2.1.3. Casting of agarose gel

1. 5µl of DNA sample was taken from the stock and 2.5µl of Bromo-phenol dye was added and mixed to be used for gel electrophoresis separation.
2. 0.8% agarose solution in 1X TAE buffer for 100 ml was prepared. The agarose was heated in microwave oven to dissolve completely, and later cooled to 40˚C, ethidium bromide solution (0.5µg/ml) was added, swirled gently and later poured into the cast and then comb was inserted.
3. When the gel was set, the comb was removed carefully and gel was kept in the gel electrophoresis unit.

3.2.1.4. Running the gel

1. The gel electrophoresis tank was filled with 0.5X TAE buffer. After placing the gel, DNA samples were loaded.
2. 75 volts current was applied for 1½ to 2 hours.
3. Gel was observed under UV light. A zigzag pattern of a single band indicated intact plant DNA.

3.2.1.5. DNA quantification

1. DNA concentration in the sample was estimated by recording absorbance at 260 nm using UV/ VIS spectrophotometer.

2. 5µl of DNA sample in a quartz cuvette was taken and the volume was made up to 1 ml with distilled water or TE buffer.
3. The absorbance of the solution at 260 and 280 nm were measured.
4. The ratios A_{260nm}/A_{280nm} were calculated.
5. DNA concentration was calculated using the relationship for double stranded

   DNA (1 O.D. at 260 nm = 50µg/ ml).

$$\textbf{Total quantity of DNA (g/ l)} = \frac{\textbf{O.D. at 260nm x 50 x Dilution factor}}{\textbf{1000}}$$

$$\text{Dilution factor} = \frac{\text{Volume made}}{\text{Volume of the aliquot}}$$

$$\text{Here, Dilution factor} = \frac{1000 \text{ l}}{5 \text{ l}}$$

$$= 200$$

3.2.1.6. Bulk Segregant Analysis (BSA)

The DNA of the two parents, IIHR-500A and IIHR-575 were pooled into two groups or bulks. The IIHR-500A plants were bulked and named as resistant bulk while IIHR-575 plants were bulked and named as susceptible bulk and bulk segregant analysis was performed as suggested by Michelmore *et al.* (1991).

3.2.2. Marker analysis

The DNA of resistant and susceptible parental population and their respective bulks were subjected EST-SSR primers, Genomic SSR

primers and SSR primers identified and QTL detected in tomato were subjected to PCR amplification to identify polymorphic markers linked bacterial wilt resistance in brinjal population.

3.2.2.1 PCR amplification using EST-SSR primers

The PCR conditions for EST-SSR marker analysis were optimised for brinjal. Optimisation of reaction conditions should precede the actual EST-SSR analysis so as to get reproducible results. Following optimisation is essential:

1. Template DNA concentration
2. *Taq* DNA polymerase concentration
3. Mg^{2+} ion concentration
4. Primer concentration
5. Primer annealing temperature
6. Primer suitable for detection of polymorphic loci in the taxa to be analysed

All the reactions were set up under contamination free conditions, as PCR is very sensitive. Therefore the stock solutions were opened only under the laminar flow hood or on a clean table. Only autoclaved micro-tubes, pipette tips, double distilled sterile water were used.

Reagents

1. Reaction buffer (10X in 100ml):
   a] Tris HCl 100mM
   b] KCl 800mM
   c] MgCl2.6H20 20mM
   d] TritonX 1%
   e] Gelatin 1%
   (pH adjusted to 9.0)

2. Primers: Stock 5µM
3. *Taq* DNA polymerase: Stock 3U/µl
4. Template DNA: Stock 20ng/µl
5. dNTPs: Stock 1mM (100µl of each dNTP pipetted out and diluted to 1000 µl)
6. Bromo phenol blue: *Stock solution:* 0.25% in 50% glycerol

   (*Working solution:* 200µl stock + 200µl of 50 glycerol)

Protocol

1. The thermo cycler (Technie-RS 232 Thermal Cycler, England) was switched on at least 15 minute before to carry out the PCR.
2. The master mix was pipetted out accurately using appropriate auto pipettes into sterile 0.5ml micro tubes.

The reagents required for PCR as follows:

Reagents	Volume (µl)
Reaction buffer(10X)	2.5
dNTPs (1mM)	2.5
$MgCl_2$ (20mM)	2.5
Template DNA(20ng/µl)	2.5
Taq DNA polymerase (3U/µl)	0.33
Primer (5 pM) for Forward and Reverse each	2.5 2.5
Water	9.67
Total reaction volume	25.0

3. Mixing was done by repeated pipetting and the contents spun down for 15 seconds at 5000 rpm.

4. The tubes were placed firmly in the wells of the thermo cycler and the following touchdown PCR program was optimised in brinjal:

Step 1: 95° C for 5 mins	Step 5: 94° C for 30 sec
Step 2: 94° C for 30 sec	Step 6: 55° C for 30 sec
Step 3: 60° C for 30 sec	Step 7: 72° C for 1 min
Step 4: 72° C for 1 min	(*Repeat step 5 to 7 for 30 cycles)*
(*Repeat step2 to 4 for 11cycles*	Step 8: 72° C for 10 mins
with a decrease of 0.5˚C per cycle)	Step 9: 4˚C hold

5. At the end of the run the tubes were taken out, 2.5µl of loading dye was added and spun for 2-5 seconds at top speed in micro centrifuge and stored at 4°C till electrophoresis.

Analysis of EST-SSR primers

Amplification profiles of 39 primers in brinjal population were scored for detection of polymorphism. The Table 3 shows the EST-SSR primers used in this study.

Agarose gel electrophoresis

The amplification products of were separated on 3% agarose gels, visualized by staining with ethidium bromide and viewed under UV light.

Protocol:

1. The gel tray was set by taping the open ends, and placed on a level surface.

2. Agarose gel (3%) was prepared in 1X TAE buffer by boiling and cooling to 40 ^{0}C. Ethidium bromide solution (0.5 g/ml) was added.

Table 3. List of EST-SSR primers

Sl. No.	SSR Loci	Repeat Length	Forward Primer (5'-3')	Reverse Primer(5'-3')
1	EEMS06	(T)14	TCATGCGAAGATTAATTAAATGTGA	GAGTGGATGATCAAGAATGGC
2	EEMS07	(T)13	CCATGCCAGAATGGAAACTT	AACGAAAACACGATCAACCC
3	EEMS10	(A)20	TCAAGCAGAACGAAGATGGA	GTAGGGGACGTGGATTCAGA
4	EEMS12	(A)16	CGGGCAACTCTTCACATTTT	ATTGGTTTGCTATCGAATTTCT
5	EEMS13	(A)14	TGAGATACGCGTACAATGACTTC	GGGGTTTTGCTGCTGTTATC
6	EEMS14	(A)13	GGAATGGACCAAACCCCTAA	AGAGCTTCGTTGCTTGGTGT
7	EEMS15	(C)12	GGGACAAATCTGACCTTTGG	CTGGTGGCAAATTCTTCGAT
8	EEMS16	(AC)7	CAATTTTTCGGTTCACTAATCAAG	CTTCAAGGAAAAAGGAGGCC
9	EEMS17	(CA)8	TGACATGTAGCTGGGCAGAG	TGGAGTGTGCATCCCAAATA
10	EEMS18	(AG)7	GGAGAAACTGAAAAATTTGTAGAGAG	GAGGAGTTTCCGACATGAGC
11	EEMS19	(AT)9	GGCATGACAAAATCATACAAACA	TGTTGGTTAAGTCCATGGGAA
12	EEMS20	(AT)8	AACATCAGCCAGGGTGTTTC	TGCTGAAAATTACAAGCCAAA
13	EEMS21	(AGA)5	TGATGTTGAACCGACACAAGA	CGTCTTCATCTTCCTCCTCG
14	EEMS22	(AAG)5	GAAGGACGTTGGTCCTGGTA	CTGTTCATTATCCCCATCGC
15	EEMS23	(TTC)5	CACCAATTTCCCCCTTCTTT	CGGTTGGTAAAGAAAACCCA
16	EEMS24	(CTT)5	CACCTGTTTGAGCACCTTGA	CACCGAAGGCAGAGAAGAAG
17	EEMS25	(CTT)5	CCCATAGCTTTGCTCGAGAT	GCACCAAAGGCAGAGAAGAA
18	EEMS26	(CTT)5	GACACTCCCCTACTTCCACCT	CGCTTAGCAGAAGCCGATAA
19	EEMS28	(TAA)21	GACGATGACGACGACGATAA	TGGACTCACAACTCAGCCAG
20	EEMS29	(ATG)5	TCAGTCAACTGCATCACCAGA	ATTCCCATTATTGGCTGCTG
21	EEMS30	(TAC)5	TTTACATGACAGCACCAGGC	ATTTTATGGGAATGGGGTCC
22	EEMS31	(TGG)5	GAGAAGTTGGCTTCAGTGCC	TAAACTCAAGGGATGCTGGG
23	EEMS32	(TCA)5	TAAGGAGTCTGATGCCGCTT	GTAATGCTCCTCCACGGCTA
24	EEMS33	(TCA)5	CTATCTCCTTTTCCCCGACC	ATGAATAAGCTGCCACCACC
25	EEMS34	(TCA)5	GCTTGATTCCCCACAAAGAA	GTTTCATCGCCCTCATCATT
26	EEMS35	(TCA)5	ATGGCTTCTGATGGACCAAG	CACTTGATGAACGTGGATGG
27	EEMS36	(TGT)5	TCTATCATCCCCAGATCCCA	AAGGTCGCATGGACATTAGG
28	EEMS37	(TCC)5	CCCTTCCTACCCACACTTCA	GTTTTGCACCTTTCCATCGT
29	EEMS38	(CAC)5	TTCAATCGAACTTCGGAACC	ATGACGGTGGATCTCGCTAC
30	EEMS39	(CTG)5	GGAGAGATGGATGCCGAATA	TCTCGACCTTAGCCTGCATT
31	EEMS41	(GCA)5	ATTCTGCATTCATCGGAAGG	GGATTGCTTGTGGGAATATCA
32	EEMS42	(GCA)6	GCTCAGCAACCACAGTACCA	GTCCGGACTTCATCAGCATT
33	EEMS44	(GCC)5	CCTTCAAACCCTCTCCCTTC	GTGAAACGTGGTGGAGGTCT
34	EEMS45	(AGAAC)4	AGCGCTTGTCCAGGCTATAA	TTTCCACCATGAGCAAATGA
35	EEMS46	(ACCAG)6	ACCAAACGTGCATGAAACAA	GGAAATGTTGGTGGAATTGG
36	EEMS47	(GCT)5.. (TTC)5	CGAACACATTCGCAAATCAC	GCATCACAAGGATGGAAAGG
37	EEMS48	(TAA)20 (CGA)8	CAATGCAAACAATTATCATTTCG	TCGATGTTGTTGTCGTCGTT
38	EEMS49	(TA)12(G)7	TGAAATTGATCAATACCTATAAATTTA	GAAAGCCAGGATAGCATTCG
39	EEMS50	(TA)9(GA)8	AAATCCGGCCATTCTGTGTA	ACATCGTTCCGCCTCTATTG

Agarose solution was poured into the gel tray with the comb in place, avoiding air bubbles and allowed to set for 20mins.

3. After removing the comb the gel was placed in the electrophoresis tank containing 0.5 X TAE buffer till the gel was fully submerged.
4. 25 µl sample of PCR sample and transferred into the wells and 5µl of 100bp DNA marker was used to assess the size of the PCR product. The leads were connected to the power source and the gel was run at constant voltage of 75Volts.
5. The run was stopped when bromophenol blue dye reached almost 2/3 the length of the gel.
6. The gel was viewed in a gel documentation system and photographed.

3.2.2.2. PCR amplification using genomic SSR primers

The PCR conditions for genomic SSR marker analysis were optimised for brinjal. Optimisation of reaction conditions should precede the actual genomic SSR analysis so as to get reproducible results. Following optimisation is essential:

1. Template DNA concentration
2. *Taq* DNA polymerase concentration
3. Mg^{2}+ ion concentration
4. Primer concentration
5. Primer annealing temperature
6. Primer suitable for detection of polymorphic loci in the taxa to be analysed

All the reactions were set up under contamination free conditions, as PCR is very sensitive. Therefore the stock solutions were opened only under the laminar flow hood or on a clean table. Only autoclaved micro-tubes, pipette tips, double distilled sterile water were used.

Reagents

1. Reaction buffer (10X in 100ml): a] Tris HCl 100mM
   b] KCl 800mM
   c] MgCl2.6H20 20mM
   d] TritonX 1%
   e] Gelatin 1%
   (pH adjusted to 9.0)
2. Primers: Stock 5µM
3. *Taq* DNA polymerase: Stock 3U/µl
4. Template DNA: Stock 20ng/µl
5. dNTPs: Stock 1mM (100µl of each dNTP pipetted out and diluted to 1000 µl)
6. Bromo phenol blue: *Stock solution:* 0.25% in 50% glycerol
   (*Working solution:* 200µl stock + 200µl of 50 glycerol)

Protocol

1. The thermo cycler (Technie-RS 232 Thermal Cycler, England) was switched on at least 15 minute earlier to carry out the PCR.
2. The master mix was pipetted out accurately using appropriate auto pipettes into sterile 0.5ml micro tubes.

The reagents required for PCR as follows

Reagents	Volume (µl)
Reaction buffer (10X)	2.5
dNTPs (1mM)	2.5
$MgCl_2$ (20mM)	2.5

Template DNA (20 ng/µl)	2.5
Taq DNA polymerase (3U/µl)	0.33
Primer (5 pM) for Forward and Reverse each	2.5 2.5
Distilled Water	9.67
Total reaction volume	25.0

3. Mixing was done by repeated pipetting and the contents spun down for 15 seconds at 5000 rpm.
4. The tubes were placed firmly in the wells of the thermo cycler and the following touchdown PCR program was optimised in brinjal:

Step 1: 95° C for 3 mins

Step 2: 94° C for 30 sec

Step 3: 60°-55° C for 30 sec

Step 4: 72° C for 1 min

(*Repeat step2 to 4 for 10 cycles with a decrease of 1°C per cycle*)

Step 5: 94° C for 30 sec

Step 6: 55° C for 30 sec

Step 7: 72° C for 1 min

(*Repeat step 5 to 7 for 30 cycles)*

Step 8: 72° C for 5 mins

Step 9: 4°C hold

5. At the end of the run the tubes were taken out, 2.5µl of loading dye was added and spun for 2-5 seconds at top speed in micro centrifuge and stored at 4°C till electrophoresis.

Analysis of genomic SSR primers

Amplification profiles of 167 primers in brinjal population were scored for detection of polymorphism. The primers are mentioned in (table 4).

Table 4. List of genomic SSR Primers used

Sl. No.	Primer_Name (CA-LR Eggplant)	Primer Sequence 5' - 3'
1	emb01C09 F	GTGCATGTCTGTGTTTGTGACTGC
2	R	GTTTATTTTATGCTGCTCCTCGGATTGAT
3	emj04D04 F	ATTGACCTAGCCCTCTTAGGCGAC
4	R	GTTTATGTTGGAAGGTTTAACCGCAGC
5	eme03A05 F	ATTTTATGCTGCTCCTCGGATTGAT
6	R	GTTTGTGCATCTCTTGTTGGTAGGAGC
7	eme01B01 F	ATGTGCATCTCTTGTTGGTAGGAGC
8	R	GTTTCTCGGATTGATCCCAAAGGGTACT
9	emi04H10 F	ATCGGAGCAAGAGACATTAGATGC
10	R	GTTTCCAACACAGTCCCCAATACAACAA
11	emh02A04 F	ATTGATTTCTAAGCGCACTCGCAC
12	R	GTTTAGGGATTGTTCAATTCTGGGTCTG
13	emb01D10 F	AAGAATCGGTCCTCTTTGCATTGT
14	R	TGCTTTTCACCTCTCCGCTATCTC
15	emf21K08 F	ATCAATGACACCCAAAACCCATTT
16	R	GTTTGAAAACCCAATACAAATCCGA
17	emi04J02 F	ACAGAAGCCTTGGCTTATATGATGA
18	R	GTTTCCCGAGAGGTTGCTACTGTAGACG
19	emg11M21F	ATAGCCTACTGCCTTCAAGACCAT
20	R	GTTTCCTACGTCCAGTCCCCTTAGGT
21	emf21M05 F	ATCCCAAGACCTGGAAGTCACCTA
22	R	GTTTAGAAGCCTTGCCACTTGGCTTAAC
23	emb01J19 F	GACAGGGATAGGGGTACGGATAGG
24	R	ATCCATGTGATGCCTCGATTTTCT
25	emb01F16 F	AAAACAGAAGCAAAGTCGGCAGTC
26	R	GTCCACCAACACCTTACCATCCTC
27	emi06A04 F	ATTTGGGACAAATGTGGGTGAGAC
28	R	GTTTCCACGCTACTTAGGGGACTCAA
29	emh11H03F	ATAACTACCTCAGCCTGTCCCCCT
30	R	GTTTGCACTTATTCCTTTTCCATTAGCTC
31	emd01D08 F	ATCAACAGGTGGCTAGGTTTCACAA
32	R	GTTTCCAGCTTAGGGCCTCCATCTACTT
33	emg11D05 F	ACGTGTGAACTTAAGCAGAATGCTC
34	R	GTTTGCAAAAAGTTTCTGTCATGCTCCA
35	emh21L21 F	ATCCATAACTACCCACACCCAACC
36	R	GTTTGATCTTTTTGACCCCAACC
37	emf01M06 F	AGCCACTTACACTGCCAACATTCA
38	R	GTTTAGTGGTAGAAGCGATGGTGGTGA
39	emb01A03 F	CGGATTTAGAGGACGTTTGGATTG

40	R	GTTTGGTGGAGCTCAGCTGTTAGTTTG
41	eme03H10 F	ATGGAATTGTTCCCTGAAGTCCTGT
42	R	GTTTCCCAAGCCTGCAATCTTTAACATC
43	emb01I13 F	AGTCGTGTAGGTCAAAGCAACTGA
44	R	GTTTGTTTCCTGGAGCAGATAGCCATT
45	ecm0I01 F	ACCTTACGCAATTTACACTTCCCC
46	R	GTTTCAATGGCGTCACCTCTCTCTCT
47	emk01B05	AGGAGGAAACACAGACACACACAA
48	R	GTTTCCCGAGCGTACAAGTAGTGAAACA
49	emf01E10 F	ACATATCCAACTGACCTCGGAAGA
50	R	GTTTAACCGCTTTGTCCCCAAATACAG
51	emk03H16 F	AGCTTGGAAATCACAAAACCCTTG
52	R	GTTTGATCGATGAATGGTGAATTGG
53	emf21P02 F	ATGAAGCAGATCTTTCGACTGCAC
54	R	GTTTAGGCCAAGGATGTCAAACTGGT
55	emh21J12 F	ACAGAACAATTCACCAGCAGTCAA
56	R	GTTTAGGAACAGGGAAAATCGTATCGGT
57	emg11M09F	ATACATTGAAATTGGCTGAGCTTG
58	R	GTTTGGATCTTCGCTAGAACTTTGGC
59	emh05B02 F	ATACCAAAGACACGTTGGGATCAT
60	R	GTTTCTAGGAGAGCATCTCCCTCCCT
61	emf11B07aF	ACGAGAGTTGCTACAGTTAAGGGG
62	R	GTTTGGGGACCAAAGTGTATTTTCAAGG
63	emf01C03 F	AGTCCACCATGAGTGAGTGAGTGA
64	R	GTTTACGTGTTGGGCCTCCAAAATATC
65	emf01A03 F	AGGAGAAGGATTAGGAAGATATCCCA
66	R	GTTTGAGAAATCGAAATGCCTCACT
67	emb01F20 F	TGGGAAAAGCAAAAGTCAGGAGAA
68	R	GTTTCATTGCAATCTCATGGAGTAGTGTTC
69	emb0048 F	TCATTGCAATCTCATGGAGTAGTGTTC
70	R	GTTTGGGAAAAGCAAAAGTCAGGAGAA
71	emk04H07 F	ATTTGGCTGGGTTGTTGGTCTAGT
72	R	GTTTGGCCCAATTACTCAAATACCCTG
73	emf21C11 F	AGGTTGGAGCCATGATTACTTGAA
74	R	GTTTGCTACCTATCAAACAGGCGGAA
75	emf01O04 F	ATCCGTTGATACTAGCCGTTGCCT
76	R	GTTTCACCCGGTATGAGTGTATCCC
77	emk04N11 F	ATCTCCCCCTCAACTTTGAACAAT
78	R	GTTTGTGTGATATAGCCCAACAATTCAC
79	emk03O04 F	ATGATTTGGGCAGCCACTTTTGTA
80	R	GTTTGGAACCAACTAAACTTAGGGCA
81	emb01H07 F	GTAATGTCGGTGGCTTGATGACTG
82	R	ACTCGGATTTCAGGAGCCACAATA

83	emf01L14 F	ACACAAGTGGAGTGGGATGACAAA
84	R	GTTTCAGCAGAAACTGCGTAGCTCCATT
85	emd0C11 F	ACGACTAAGGAGGCATTTTCTTGC
86	R	GTTTCACTTGGCAAAGCTAGCTATATTCTTG
87	emh11I06 F	ATTTCAAACCGTTCCTCTGCTCTT
88	R	GTTTGCACAATCATCAAGGCTCCTCTTT
89	emh11N11 F	ATTCAGTTCTTCGCTTTGGAGCTT
90	R	GTTTCCAAACCCGACCCATCCTAAATAA
91	emd13H06 F	ATAGTGGTTTTCCAATTACCCCCA
92	R	GTTTACATTCTACCCTCCCCAGACCC
93	eme05G05 F	ACAAGAAAGAGGAGCTGGGGAAATTG
94	R	GTTTCCTTCTTGGGAAGACAACTTATCA
95	emf01K16 F	ATTTGGACAAGAACAAGGATGGCT
96	R	GTTTCACTCACAATTCGAGACACTCGGT
97	emj03E23 F	AGCACAAAGGAGAGAAAGCAATTT
98	R	GTTTAGAAGCTGTTATCGAATCGTTGCC
99	emf11N23 F	ATGTTCTTCCCTTTTTCCCCTTTT
100	R	GTTTCCAAGAAAGAAGAAAACCCCACA
101	emf01K21 F	AGTAGGACAAACCATAAGATGCCAGA
102	R	GTTTACACCATGATGCCAAGACCCT
103	emg01A17 F	ATAAGCCAAAGCAAGCACACTTGA
104	R	GTTTGAGCTGAAGGTATGCAAGCTGGA
105	emj01G23 F	ATTAACTGGCCATGAACACCTGTC
106	R	GTTTGACCTCAATAAAGGGGGTTTGCAT
107	emi04P17 F	ACCAAGAATACAGCAACTTGAGGA
108	R	GTTTCATACCCGGCAAGTGTTTGATAA
109	emd03C01 F	ACGGGAGTTGTTTGTTGGAAGTCCTG
110	R	GTTTCCAAATTTTTGGGTCGTGACAGTT
111	emf11L21 F	ATAGCCTAGGTAACGTACCCCTCG
112	R	GTTTGGCTCTATTTCCTGGGCTTTTCAT
113	emf21N03 F	ACCAGAGGAGCAAAGGGAAAAATA
114	R	GTTTACGCTACTGGACCAAACCAACAAT
115	eme25D01 F	AGTCCCAACCAAAATCGTAGAGGC
116	R	GTTTCACTGAAGGATGTGGAGTGTGA
117	emf11D18 F	AGAGACAGGGAGAGTGCATTCTATG
118	R	GTTTGCAGTTCATAAGGTTGCATCAATAC
119	emf11H23 F	ATTCTGAAAACAAGAGCAGCCCTC
120	R	GTTTCTCAACACCTCTGTGTCTGGCAT
121	emi03M03 F	ACTGTGTGTGTGTGTGTGTTGA
122	R	GTTTAGGTCCAAATACATTCGACCCTCC
123	emg11I04 F	ACATTCAGGTTTCTCTCTTCCCCTC
124	R	GTTTGCCCTCCACTCTTGATCATCTTT
125	emf21I04 F	AGAGAGGTGACTGAAAGCAAAACG

126	R	GTTTGGAAAATACCAACCAAGCCAATCA
127	emg11A06 F	AGTGCTAATATGCAAGGGGAATGG
128	R	GTTTACGGTGATCTTTCCGTATTCCAAA
129	emg11D19 F	ACATGGTAGGTGGAGACAGAACCA
130	R	GTTTGCAAAGAGCCTATGACCAGGTT
131	emg11D19 F	TCATGGTAGGTGGAGACAGAACCA
132	R	GTTTGGATTAGCATGTGGAGGACTGAA
133	eme08D09 F	ATGGATTAGCATGTGGAGGACTGAA
134	R	GTTTCATGGTAGGTGGAGACAGAACCA
135	emf21A23 F	AGATTTGGTTGCTATAGTTAGGGTT
136	R	GTTTAGGAGAGAGGTGAGCGAGATCAAA
137	emf21I02 F	AGTGCATTTCTCAAATCAAAAGGG
138	R	GTTTCAATTTCACAGGCTCCTGCATTA
139	emf21H22 F	ATCAAGATGAACAAGACTAAGGAGTGC
140	R	GTTTCTTCAACCTGTCTTTAGCCCA
141	emh02E08 F	AGGCGTTCAGCAGAGAAGAAATTA
142	R	GTTTGCTTCCTTAAGTGGCATCTGAAA
143	emf21E10 F	AAGAGAGTCGAGAGAGGTGGATTG
144	R	GTTTCTCCTTTGCAATCTCTTTTCA
145	emf01D24 F	ATACAGTGCCCAACACGATTCAAG
146	R	GTTTCAGATAGATGGAAATTAAGGGGGTG
147	emd01E11 F	ACAGGTCTACCACGAGGCATCTTCT
148	R	GTTTGCCCCCAATCCATTAGTGTTAAG
149	emf01G17 F	ATGGCAACTGATAATGCAGACGTG
150	R	GTTTCTCACTCTTACATGTGGCTGGC
151	ecm009 F	ATCTAGTACCATCAAGTCTAAGCAGCA
152	R	GTTTAACAACAGCTGAGGCCATGAAA
153	eme11f04 F	ACCCCCAAATCAAATCATTTACCC
154	R	GTTTGGCATGGTTAGGGTTTTTAGCGTT
155	emd12B05 F	ACGGAGTAGGCTCGGAGCGTGATATT
156	R	GTTTGAAAGGGCAAAAAGTCCAAACAAC
157	emb01L13 F	TCAAAAGACTTGAAACCCGATGGT
158	R	ATCAGGTTTTTGATCACCGGACA
159	emg21A08 F	ATGGCAAGGACTGAGGTATCACAA
160	R	GTTTCCGCTTATTTGATGGATCTTGC
161	emg01D17 F	ACTAATAGCCAAAACACCCCACAA
162	R	GTTTAGTATGTGACCTTGCGCTGGAAA
163	emj03E03 F	AGACAAGCTTTGAGTGTGGAGTGA
164	R	GTTTCTCACCTTTTGCAACAGTATCCG
165	emg21C17 F	ATTTCCCAGCTGCTTGTTGATCTT
166	R	GTTTGCTTTGATTGATGCTATCTGGGA
167	emd03D09 F	ACAGCACTGCTCTAATGGCTTTGGTC
168	R	GTTTCAAGTGTGGGGGTGGACTACACTTA

169	emf11M07 F	ATCTCTCTCACTGAACCTACTTTGC
170	R	GTTTGGAGAGTGAACATCTGCCATTGT
171	emf11F07 F	ATTGGACCAAGTATGTGGTGTGGA
172	R	GTTTGCTCTTCACCTGGCTTGATTT
173	emh01E15 F	ATGACCCTGAGTGTGTGATCCGAG
174	R	GTTTGGTTTGATCAGGGAAAGAGAAGC
175	emf11B07bF	ACGAGAGTTGCTACAGTTAAGGGG
176	R	GTTTGGGGACCAAAGTGTATTTTCAAGG
177	emb01H20 F	TCTTGTTCCCAGTCTATCGCTAATCA
178	R	ATCCGAATTTAGTCGGGCTTCAAT
179	emi03K06 F	ATGTTTTGTGGTGCCACGTAGATG
180	R	GTTTAAGGTGCAGGGTAATTGTCATTGC
181	emh01C16 F	AGATCATGTTCCCTCACCAAGAAA
182	R	GTTTGAGCAAGCCTGTGGAATCTAGG
183	emi02F16 F	ACAAGCTTGAACATCCTTCGGGTA
184	R	GTTTGAAATCACATCATGTCCTCACTC
185	emd05F08 F	ACAGGCAACCAAGTTACCAACCCTTT
186	R	GTTTAAAATGTCCGGTTATGGATTCGC
187	emg11I03 F	ATTAGGCACAAGTGCCACCTGAAT
188	R	GTTTCAGCCGGGAGTCTGATAGGTAAAA
189	eme36B11 F	ATAGCCAATTACCCTAAGCCACAG
190	R	GTTTGACGGTCGTCTTTTGTATTCAGGC
191	emj03A17 F	ACTAAAGACAAATCCTGATGAGATG
192	R	GTTTGGCTAAGACCACCGAAAACAAC
193	emh11E08 F	ATTTACTATGCTACTTCACACCCACC
194	R	GTTTACTGATCGCAGGAAAAGGGAAAG
195	emb01N07 F	TGATAAGAAGGGCAAGCTCAGTCC
196	R	GTTTCGAGCTTATGGCTACACTGGACCT
197	emi03G16 F	ATCCCGTATTTTTGTGCATTGGAG
198	R	GTTTGGGCCAACCTTATCAACATTC
199	emh11O01F	ATTGTGTCGATGAGATTTTGGTCA
200	R	GTTTAGCTACGTTGGTTTGGTGCTGAA
201	emd21E01 F	AGGTATCGAAGAGAGTGAATGCCT
202	R	GTTTCCCCATTTCATCTGAAAAATCCAC
203	emf11F24 F	ATGGTTTTGATATGGTGGCAGGAA
204	R	GTTTCTCTGGAGCCACACCTACACAG
205	eme09E09 F	ACGGTATCGAAGAGAGTGAATGCCT
206	R	GTTTCCCCATTTCATCTGAAAAATCCAC
207	emd18C06 F	ATTTCTGAGGTTTAACATCGCCGT
208	R	GTTTCGGAGGAGAGCAAGTTCTGCTTTA
209	emb01E02 F	GAACCCGGTTGCTTTATCTTAGCC
210	R	GAACCCCAAACAAGCCTCATAACA
211	emi06F08 F	ATAATGAACCAAAGCGAGAGCAAC

212	R	GTTTCAGGTCCATAGGGGTGGATCTATG
213	emb01O01 F	TTAACATCGCCGTTGGCTTCTTAG
214	R	GTTTCGATAACCAAAAGGGGTTTCAACA
215	emi02E15 F	ATTGACGGTGGAAAAGGAGTTGGT
216	R	GTTTGGCGGCTTGATGATTTAAGTTTTG
217	eme03F04 F	ATATGACGACAGACGTAAAGCGACC
218	R	GTTTCAGAGTTTTGCCATCTGTGTCGAG
219	emb01D19 F	CGACCCCAGATCCAGAAATAAAGA
220	R	CCCAAGAGTTGTACTCGTCAACCA
221	emb01E03 F	GCGAGAATTTAAAAGGGGGAAGTG
222	R	TTGAACCGTCAAGATCCTTCCATT
223	emf21A12 F	ATCCTGGCCATGTTTCTCCATTTA
224	R	GTTTGCTTTCTAGGAGACTTTTAGCC
225	emi02E20 F	ATAGTTCCCTGAGTCCCCATTTGA
226	R	GTTTCAAGACTGAAGGACCACAAATCACA
227	emi02K11 F	AGGGGCTAGCAATAGTCTTCGAGT
228	R	GTTTCAAACGTTGGCCATGTACTCT
229	eme04G05 F	ACAGTTCGTTTAGGAGTCTTTCCTTTG
230	R	GTTTCACTGAATTGAATTGGGTCCATGA
231	emg21I10 F	ATCCTTGTTTCTTGCAGGGACTTG
232	R	GTTTGGTCTCTTTGGTTTTGCTAGTGG
233	emf01A06 F	ACATCATACGAAAGCCCTTAAGCC
234	R	GTTTAAGTGCCCTCTCAGAAAGAAGCCT
235	emh05H12F	AGTCACTGCTCTTAGTTTCTGCAA
236	R	GTTTCAGAGCAGCGATCCTTTCTTCATT
237	eme36B08 F	ACCTGTCCCATCTTCTGATTCATT
238	R	GTTTCTAGAAAAGTCCCAGCCAACA
239	emb01F01F	AAAGGAGGAAAGGGAAAGGGAAAG
240	R	AATAAAGCCTGAGAGGGGAAGACG
241	emb01G19F	AATTAAGGCTGAGAGGGGAAGACG
242	R	AAAGGAGGAAAGGGAAAGGGAAAG
243	emb01O09F	GTCCTACGTCCAGTCCCCTTAGGT
244	R	TCCCTTGGAATGCCATCTCTTTTA
245	emd04E10F	ATGGTTATCCGGCCTCCTATACCTC
246	R	GTTTAGGAATTGAAGGTCCAAAGCATGA
247	emd05B11F	ATTGCTTCAATTAAGGCTGAGAGGG
248	R	GTTTATTTTTCGCTTGAGAGTGGTGGC
249	eme01D03F	ACAAGAATCGGTCCTCTTTGCATTGT
250	R	GTTTGCTTTTCACCTCTCCGCTATCTC
251	emf01O01F	AGGAATTGGATTTCCACTCATACG
252	R	GTTTGGAAGATGAGATTCCTTTCTTGA
253	emf21N12 F	AGTCAATAACCTCCCACATCCCAC
254	R	GTTTGCTTGAGCACCATGTGTTTGAT

255	emg11B20F	ATCATTGCCGTATCAGGTTCACTC
256	R	GTTTGGGAAAGTTGAGAATTTCTTGGGG
257	emg11P03F	ACTCGCCTCTCTCAATCTTTCTTG
258	R	GTTTCAATATAACCTCGGCTATGAGACCC
259	emg21G24F	ATAAATCCACCAGACCAGCAAAAC
260	R	GTTTCAGTTATCCCCCTTCTGTTCCCTC
261	emi02C21 F	AGTGAGGAGAAGAATCAGAGGATCA
262	R	GTTTCGCGACTAAGTTTTGTTCCTGAAA
263	emi03L23 F	ATCCACAGGGTGATGATAAATGTTG
264	R	GTTTCATAGCTTGGCACCAAAAACG
265	emk02K24F	AGTAGGCTAAACGACCTCTAAATTTGC
266	R	GTTTGGTGTTGACAAAAAGAACCTGAC
267	emi04O04 F	ATTAAGGGCTTCCATCACTTTGGA
268	R	GTTTGCACCATCTATCAATCCACGA
269	em135 a F	ATCCTGTTGCTGCTCATTTTCCTC
270	R	AGGAGGATCCAAGAGGTTTGTTGA
271	em245 b F	CCAACCGCTGCTAATTTCTCCTCT
272	R	ATTTGAAGCTCCATTGTTGGGACA
273	emd01B12F	ATCAATAGCTTGCCACTTGGCTTAAC
274	R	GTTTAGGAAACCTAACCCAAACCTGGAA
275	eme03H04F	ACGCCCGTCGTAAACTTTCCATGATA
276	R	GTTTGTGCAGCACATTCTAGCGACACTT
277	emf01B07 F	ATACCAGATAAAACGAAGCACGGC
278	R	GTTTGATGAGTGTGGGACTTGAAGA
279	emf21M21F	ATGTCTTGGACCCCGCTAAGTATC
280	R	GTTTCGCGGAAGCAATTAAAAATGAC
281	ecm031 F	ACCAAAGGAGAAGGAGATGTGCTA
282	R	GTTTGCTTCCCTTCCAAAATGACAAG
283	em4_1 a F	GCATACAGCAAATCCCAGCAAATA
284	R	TGTGAGTATGGGATTCTGGTCGTT
285	emd01B12F	ATCAATAGCTTGCCACTTGGCTTAAC
286	R	GTTTAGGAAACCTAACCCAAACCTGGAA
287	ecm090 F	AGCATCTGTCACTCTGTACCTGGA
288	R	GTTTCTGCCATGCTAATGGACAAAGAA
289	em155 a F	CAAAAGATAAAAAGCTGCCGGATG
290	R	CATGCGTGAGTTTTGGAGAGAGAG
291	em256 b F	TCTTAAATGTGCCAGCCCTCTCAT
292	R	GCTGCTGTTTGTTGACAAAATTGG
293	em119 a F	CCCCACCCCATTTGTGTTATGTT
294	R	ACCCGAGAGCTATGGAGTGTTCTG
295	ema0060 F	GCTCGGTATGGCATAAGTTTGGAG
296	R	GTTTAGCTTCCCCATTGTACCCCTGAAC
297	emb0001 F	CCTACCCAAACCCTGCATACAAAA

298	R	AGGTTTGTGGAATCCTGTGCGTAT
299	emb01O20F	AAAGGCGGGGACAAGAGATTGTAT
300	R	ATCGTTCTCCCTCTGTTCATCCTG
331	ecm070 F	ATCAAAATCCATGGAGGTTTTCCA
332	R	GTTTCCTGTTCCGATTTAGCTCTCACC
334	em117a F	GATCATCACTGGTTTGGGCTACAA
335	R	AGGGGAGAGGAAACTTGATTGGAC
336	em236 b F	TGGCACTGGAAATGCTTATTGATG
337	R	CAAATTACGATAGCGCCAAACGAT
338	ema0008F	TGTGCTTTGCTAATGTGGGTCTTT
339	R	TCCTGCATGAAATGTGTTTGAGTG
340	emb0015F	CCTTGCTTTTTGTGATGCAGATTG
341	R	CTCGTCTCATGGAGCGATATTGTG
342	emd01C11F	ACGACTAAGGAGGCATTTTCTTGC
343	R	GTTTCACTTGGCAAAGCTAGCTATATTCTTG
344	em134 a F	AGTAAGGGAAAGTGCTGACGAAGG
345	R	CAGAGTCATCGTTATGGGGAGGTT
346	ecm023 F	ACCAAACAGTGTGACAAAGCATGA
347	R	GTTTCCCACTTGGGTATCACTAAGCCAC
348	emd01A01	ACAGCAAAATGCCTAATGACAGCACA
349	R	GTTTATGCCTGACTCTGCTTGTGCCTA
350	em114 a F	AGCCTAAACTTGGTTGGTTTTTGC
351	R	GAAGCTTTAAGAGCCTTCTATGCAG
352	em120 a F	GGATCAACTGAAGAGCTGGTGGTT
353	R	CAGAGCTTCAATGTTCCATTTCACA
354	em232 b F	CTCCCCTTAAATTAGCCCATCCTG
355	R	AAAAAGGTATGCTGCCCCTGTTTT
356	ecm032 F	AGCATGCAAAAGAGTGAGCCTAAG
357	R	GTTTCCCTCTCTACTGTGCCCATGATTT
358	em206 b F	ATCTTAATCTTCCTGCTCCTGTTG
359	R	CTGGAAACAAGCTCGCTACCAAAT
360	emd01C04F	ACCTGCATGAAATGTGTTTGAGTG
361	R	GTTTGGGTCTTTTCATCTCAAAATGGG
362	em140 a F	CCAAAACAATTTCCAGTGACTGTGC
363	R	GACCAGAATGCCCCTCAAATTAAA
364	em21_7 a F	TGCAGTAGTCTCTGGCTGTGTCTG
365	R	CCAGATGCTTTGAGGTGAGAGATG

Agarose gel electrophoresis

The amplification products were separated on 3% agarose gels, visualized by staining with ethidium bromide and viewed under UV light.

Protocol:

1. The gel tray was set by taping the open ends, and placed on a level surface.
2. Agarose gel (3%) was prepared in 1X TAE buffer by boiling and cooling to 40 ^{0}C. Ethidium bromide solution (0.5 g/ml) was added. Agarose solution was poured into the gel tray with the comb in place, avoiding air bubbles and allowed to set for 20mins.
3. After removing the comb the gel was placed in the electrophoresis tank containing 0.5 X TAE buffer till the gel was fully submerged.
4. 25 µl sample of PCR sample and transferred into the wells and 5µl of 100bp DNA marker was used to assess the size of the PCR product. The leads were connected to the power source and the gel was run at constant voltage of 75Volts.
5. The run was stopped when bromophenol blue dye reached almost 2/3 the length of the gel.
6. The gel was viewed in a gel documentation system and photographed.

3.2.2.3. PCR amplification using Tomato *Ralstonia* QTL SSR primers

Bacterial wilt in tomato is a major issue. The QTL that confers resistance to bacterial wilt in tomato was identified and mapped by using SSR markers in cultivar Hawaii 7996 (Hai, 2007). Since tomato and Brinjal belongs to same family, the genomic SSRs linked to bacterial wilt in tomato were used to validate in brinjal genotypes.

The PCR conditions for tomato *Ralstonia* QTL SSR marker analysis were optimised for brinjal. Optimisation of reaction conditions should precede the actual genomic SSR analysis so as to get reproducible results. Following optimisation is essential:

1. Template DNA concentration
2. *Taq* DNA polymerase concentration
3. Mg2+ ion concentration
4. Primer concentration
5. Primer annealing temperature
6. Primer suitable for detection of polymorphic loci in the taxa to be analysed

All the reactions were set up under contamination free conditions, as PCR is very sensitive. Therefore the stock solutions were opened only under the laminar flow hood or on a clean table. Only autoclaved micro-tubes, pipette tips, double distilled sterile water were used.

Reagents

1. Reaction buffer (10X in 100ml): a] Tris HCl 100mM
   b] KCl 800mM
   c] MgCl2.6H20 20mM
   d] TritonX 1%
   e] Gelatin 1%
   (pH adjusted to 9.0)

2. Primers: Stock 5µM

3. *Taq* DNA polymerase: Stock 3U/µl

4. Template DNA: Stock 20ng/µl

5. dNTPs: Stock 1mM (100µl of each dNTP pipetted out and diluted to 1000 µl)

6. Bromo phenol blue: *Stock solution:* 0.25% in 50% glycerol

(*Working solution:* 200µl stock + 200µl of 50 glycerol)

Protocol

1. The thermo cycler (Technie-RS 232 Thermal Cycler, England) was switched on at least 15 minute earlier to carry out the PCR.
2. The master mix was pipetted out accurately using appropriate auto pipettes into sterile 0.5ml micro tubes.

The reagents required for PCR as follows

Reagents	Volume
Reaction buffer (10X)	2.5µl
dNTPs (1mM)	3.0µl
$MgCl_2$ (20mM)	0.6µl
Template DNA (20 ng/µl)	4.0µl
Taq DNA polymerase (3U/µl)	0.40µl
Primer (5 pM) for Forward and Reverse each	2.5µl 2.5µl
Distilled Water	9.50µl
Total reaction volume	25.0 µl

3. Mixing was done by repeated pipetting and the contents spun down for 15 seconds at 5000 rpm.

4. The tubes were placed firmly in the wells of the thermo cycler and the following touchdown PCR programme was optimised in brinjal:

Step 1: 95° C for 3 mins

Step 2: 94° C for 30 sec

Step 3: 60°-55˚ C for 30 sec

Step 4: 72° C for 1 min

(*Repeat step2 to 4 for 10 cycles with a decrease of 1˚C per cycle*)

Step 5: 94° C for 30 sec

Step 6: 55° C for 30 sec

Step 7: 72° C for 1 min

(*Repeat step 5 to 7 for 30 cycles)*

Step 8: 72° C for 5 mins

Step 9: 4˚C hold

5. At the end of the run the tubes were taken out, 2.5µl of loading dye was added and spun for 2-5 seconds at top speed in micro centrifuge and stored at 4°C till electrophoresis.

Analysis of Tomato *Ralstonia* QTL SSR primers

Amplification profiles of 71 primers in brinjal population were scored for detection of polymorphism. The primers are mentioned in (table 5).

Agarose gel electrophoresis

The amplification products were separated on 3% agarose gels, visualized by staining with ethidium bromide and viewed under UV light.

Protocol:

1. The gel tray was set by taping the open ends, and placed on a level surface.

2. Agarose gel (3%) was prepared in 1X TAE buffer by boiling and cooling to 40 ^{0}C. Ethidium bromide solution (0.5 g/ml) was added. Agarose solution was poured into the gel tray with the comb in place, avoiding air bubbles and allowed to set for 20mins.

Table 5: List of Tomato *Ralstonia* QTL SSR Primers used

Sl. No.	Oligo Name	Primer sequence 5'-3'
1	emh11G09F	5'ACTCTGTCATTATTTTTGGTCC
2	R	5'GTTTCATCCACTTGGGCTTAAAATGCT
3	emh01J23F	5'ATGCAGCTCCCATAAACCCTAAAA
4	R	5'GTTTCCAAGACCAGCACTCCAAAC
5	emh05B12F	5'ATAATTGGACCAAGTGTGGTGTGG
6	R	5'GTTTAGGGGCCTCGATATAGTAGGTTGG
7	emh05B13F	ATAATTGGACCAAGTGTGGTGTGG
8	R	GTTTAGGGGCCTCGATATAGTAGGTTGG
9	emf01M12F	ACGCACCTCAGACTGAAGAGTTTT
10	R	GTTTGAAAGGGAATTGAATGGTGCTTCA
11	emg21k22F	ATGACTAACATGGCTGAGTGCCAA
12	R	GTTTCAAACCCTACTTTGTGGGACTTCG
13	emf01P21F	ACACCGCTTCTAGGTTTTGCACTC
14	R	GTTTGATGGCACCTTGTGCTCCTAT
15	emf01P22F	5'ACACCGCTTCTAGGTTTTGCACTC
16	R	5'GTTTGATGGCACCTTGTGCTCCTAT
17	emd18E02F	AGTGCTCTGAACTCCTTTCCTTCA
18	R	GTTTCCCTAAAAGGAATATGTGCTCTGG
19	emd18E03F	AGTGCTCTGAACTCCTTTCCTTCA
20	R	GTTTCCCTAAAAGGAATATGTGCTCTGG
21	emd18E04F	AGTGCTCTGAACTCCTTTCCTTCA
22	R	GTTTCCCTAAAAGGAATATGTGCTCTGG
23	emh01O20F	ACTGCAATATTTGGGTTGCAGAGA
24	R	GTTTGAGGTCATTTGGTGGTTCAGGT
25	emh01O21F	ACTGCAATATTTGGGTTGCAGAGA
26	R	GTTTGAGGTCATTTGGTGGTTCAGGT
27	emh01O22F	ACTGCAATATTTGGGTTGCAGAGA
28	R	GTTTGAGGTCATTTGGTGGTTCAGGT
29	emh01O23F	ACTGCAATATTTGGGTTGCAGAGA
30	R	GTTTGAGGTCATTTGGTGGTTCAGGT
31	emg11K23F	ATGACTGCAATGTCCTCACTTGGT
32	R	GTTTGGGGGCTATTTTTAAAACTGGATAG
33	emg21F05F	ATATTGAAGAGGGGAGAATGAGCA
34	R	GTTTAAGAGGGAAGATTTTGGGGCCTA

35	emf11N03F	ATATCGTTGAAGCAAACATCTGCC
36	R	GTTTCGCTATATCACCCCAATCA
37	emf11N04F	ATATCGTTGAAGCAAACATCTGCC
38	R	GTTTCGCTATATCACCCCAATCA
39	emf11N05F	ATATCGTTGAAGCAAACATCTGCC
40	R	GTTTCGCTATATCACCCCAATCA
41	emf11N06F	ATATCGTTGAAGCAAACATCTGCC
42	R	GTTTCGCTATATCACCCCAATCA
43	emd16C09F	ATTCACTTTCCTTATTTTTGGTCGG
44	R	GTTTATGCAGCTGGTATATCTGGCAA
45	emd16C10F	ATTCACTTTCCTTATTTTTGGTCGG
46	R	GTTTATGCAGCTGGTATATCTGGCAA
47	emd16C11F	ATTCACTTTCCTTATTTTTGGTCGG
48	R	GTTTATGCAGCTGGTATATCTGGCAA
49	emf01P24F	ATAGCAATGTAGACCTGTAGCGCA
50	R	GTTTCCAACTCTCCATCCTGTCATTCTTG
51	emf01P25F	ATAGCAATGTAGACCTGTAGCGCA
52	R	GTTTCCAACTCTCCATCCTGTCATTCTTG
53	emf01P26F	ATAGCAATGTAGACCTGTAGCGCA
54	R	GTTTCCAACTCTCCATCCTGTCATTCTTG
55	emf01P27F	ATAGCAATGTAGACCTGTAGCGCA
56	R	GTTTCCAACTCTCCATCCTGTCATTCTTG
57	emf01P28F	ATAGCAATGTAGACCTGTAGCGCA
58	R	GTTTCCAACTCTCCATCCTGTCATTCTTG
59	emf01P29F	ATAGCAATGTAGACCTGTAGCGCA
60	R	GTTTCCAACTCTCCATCCTGTCATTCTTG
61	emf01P30F	ATAGCAATGTAGACCTGTAGCGCA
62	R	GTTTCCAACTCTCCATCCTGTCATTCTTG
63	emf01P31F	ATAGCAATGTAGACCTGTAGCGCA
64	R	GTTTCCAACTCTCCATCCTGTCATTCTTG
65	emd02A05F	ACCATTGTACCCCTGAACGGATATACA
66	R	GTTTCCGATGTACAGTTTGTTGACCCAC
67	emd02A06F	ACCATTGTACCCCTGAACGGATATACA
68	R	GTTTCCGATGTACAGTTTGTTGACCCAC
69	emf11A03F	ATACATTCTACCCAACTATCCTTCCA
70	R	GTTTGTTCGTACTTCATCGTGTTGGC
71	emf11A04F	ATACATTCTACCCAACTATCCTTCCA

72	R	GTTTGTTCGTACTTCATCGTGTTGGC
73	emf01J09F	ATAGCACCCACACTAAACCTTGGG
74	R	GTTTCACTTCTTGGTCCATTGTTCAGA
75	emi02F22F	AGGACTAATTACGAGATTTGGGGC
76	R	GTTTACTCTTTTGCCCTTGATCGTTCC
77	eme02B08F	ATTGGGAAAAGCAAAAGTCAGGAGA
78	R	GTTTGGGTTCCTCATAATTGTAATGGC
79	eme02B09F	ATTGGGAAAAGCAAAAGTCAGGAGA
80	R	GTTTGGGTTCCTCATAATTGTAATGGC
81	emg1l17F	ACAACATTTCTAAGGGCCTTCACG
82	R	GTTTGGGCATATTTGGCACTTGTTGAAT
83	emb01K07F	AACTGCAATAAGGCTTGGGGAACT
84	R	GCTGCAGCATTTCTAAACTCACGA
85	emb01K08F	AACTGCAATAAGGCTTGGGGAACT
86	R	GCTGCAGCATTTCTAAACTCACGA
87	emb01K09F	AACTGCAATAAGGCTTGGGGAACT
88	R	GCTGCAGCATTTCTAAACTCACGA
89	emd17D06F	ATTCATTTCGGGGATGATGTTTGT
90	R	GTTTGGGACAATGAGGCTAAGGGAAAAC
91	eme11G05F	ACTCGTCTCATGGAGCGATATTGTG
92	R	GTTTCCTTGCTTTTTGTGATGCAGATTG
93	eme11G06F	ACTCGTCTCATGGAGCGATATTGTG
94	R	GTTTCCTTGCTTTTTGTGATGCAGATTG
95	emd18B04F	ATTTCTGAGGTTTAACATCGCCGT
96	R	GTTTCGGAGGAGAGCAAGTTCTGCTTTA
97	emd18B05F	ATTTCTGAGGTTTAACATCGCCGT
98	R	GTTTCGGAGGAGAGCAAGTTCTGCTTTA
99	emd13E02aF	AGTTTGAGCTTTGACTCATCCATGT
100	R	GTTTCTATTGCCACGACCCAAGTGT
101	emh03B06F	ACTTGCACTACATACCCGCCAAGT
102	R	GTTTAACCCCTCAATTTGATTACCAGGG
103	eme08A04F	ATCATTAAAGGGGTGGTACGGTTG
104	R	GTTTGAACTTGAATCGTACAAAGAGGAGTG
105	eme08A05F	ATCATTAAAGGGGTGGTACGGTTG
106	R	GTTTGAACTTGAATCGTACAAAGAGGAGTG
107	emd05D12F	ATTGGGTTTGAGAGTGGTGATAACG
108	R	GTTTCCTGAATTACCAAATTGCGCCTAA

109	eme05B09F	ATGAAAACTCCACTCTACTCTACTCCAC
110	R	GTTTGCTAACGTACGCCTCAATTGCTCT
111	eme05B10F	ATGAAAACTCCACTCTACTCTACTCCAC
112	R	GTTTGCTAACGTACGCCTCAATTGCTCT
113	emd07A07F	AGGACTTCTTGGAAGGAGAGGAAC
114	R	GTTTCCATTTGGGCTGAAAGAAAGATTC
115	emd07A08F	AGGACTTCTTGGAAGGAGAGGAAC
116	R	GTTTCCATTTGGGCTGAAAGAAAGATTC
117	emh01J03F	AGAAATAAAGGGAGTGACATCGCA
118	R	GTTTGCAACATAGGAAGTTAAATAGCTCGAC
119	emh01J04F	AGAAATAAAGGGAGTGACATCGCA
120	R	GTTTGCAACATAGGAAGTTAAATAGCTCGAC
121	emb01C12F	AAAAAGCTCTGCCCAAACAAGC
122	R	GACTTTCCTCACTAATTCACAACCA
123	emb01C13F	AAAAAGCTCTGCCCAAACAAGC
124	R	GACTTTCCTCACTAATTCACAACCA
125	eme12G04F	ACGTGGAACCAAGCAACAAACAATA
126	R	GTTTCTAAGTTGCTGCGGGACTTTATGG
127	emh02E08F	AGGCGTTCAGCAGAGAAGAAATTA
128	R	GTTTGCTTCCTTAAGTGGCATCTGAAA
129	emh11B18F	ATCAAAACCAACCTCCAGTTCTCG
130	R	GTTTCAAATCGCAGAGTTCATCCTTCCT
131	emh11B19F	ATCAAAACCAACCTCCAGTTCTCG
132	R	GTTTCAAATCGCAGAGTTCATCCTTCCT
133	eme03B08F	ATGCCTCATCAACCTCAAAGGTCT
134	R	GTTTCTCACAAGTGGGGTCTAGGGA
135	eme01G10F	5’ACGTCTCATCCGAAATATAATGCCGC
136	R	5’GTTTGATAAGAAGGGCAAGCTCAGTCC
137	eme01G11F	ACGTCTCATCCGAAATATAATGCCGC
138	R	GTTTGATAAGAAGGGCAAGCTCAGTCC
139	eme01G12F	ACGTCTCATCCGAAATATAATGCCGC
140	R	GTTTGATAAGAAGGGCAAGCTCAGTCC
141	emh01F12F	ACACTCAGCCTTATTGCCATGTGT
142	R	GTTTGGGAATAGGAAGGAGCTGG

3. After removing the comb the gel was placed in the electrophoresis tank containing 0.5 X TAE buffer till the gel was fully submerged.

4. 25 µl sample of PCR sample and transferred into the wells and 5µl of 100bp DNA marker was used to assess the size of the PCR product. The leads were connected to the power source and the gel was run at constant voltage of 75Volts.

5. The run was stopped when bromophenol blue dye reached almost 2/3 the length of the gel.

6. The gel was viewed in a gel documentation system and photographed.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

The results of the investigation on "**Phenotyping and Identification of SSR markers linked to bacterial wilt in brinjal**" are presented in this chapter. Altogether, 39 EST-SSR, 167 genomic SSR and 71 tomato *Ralstonia* QTL SSR primers were used for screening of brinjal parental populations in search of polymorphic bands specific for resistance and susceptibility to bacterial wilt. The molecular analysis using the different primers was carried out for two diverse parents viz, IIHR-500A (Resistant) IIHR-575 (Susceptible) as well as its F_2 population.

4.1. EXPERIMENT 1: Phenotyping of brinjal genotypes for bacterial wilt resistance

A total of 310 F_2 brinjal plants were grown in polyhouse (plate 7) and inoculated with *Ralstonia solanacearum* inoculum. The disease progress interval in the F_2 population was recorded for a period of 30 days from bacterial inoculation. The first symptom of wilt was observed after 5 days of inoculation.

Symptom of bacterial wilt was observed in F_2 brinjal plant population after 5 days of inoculation. Wilting followed by death of plants was observed. Out of 310 F_2 plants, 73 plants showed symptoms of wilt over an observation period of 30 days. Rest 237 plants did not show any symptoms of wilt after *Ralstonia* inoculum treatment. The ratio of non-wilted plants to wilted plants was calculated to be approximately (3:1) (Table 6).

The calculated value of χ^2 from the data in table 7 is 0.341, while that from the χ^2 log table at 0.05 probability and 1 % degree of freedom is 3.841. Therefore, the null hypothesis is accepted, and it is concluded that the data were in agreement with 3:1 ratio.

Plate 7 : **Representation of F_2 plant population.**

Plate 8: **Individual F_2 Plant Resistant to bacterial wilt and individual F_2 Plant Susceptible to bacterial wilt.**

Table 6. Disease occurrence in F_2 plants

	No. of F_2 plants	**Percentage of disease in the population**	**Ratio**
Plants showing no disease **(resistant)**	237	76.4	76.4 **:** 23.54 (3:1)approx
Plants showing disease **(susceptible)**	73	23.54	

Table 7. χ^2 Analysis data

Trait	**O**	**E**	**E-O**	**(E-O)2**	**χ^2**
Resistant	237	232.5	-4.5	20.25	0.087
Susceptible	73	77.5	4.5	20.25	0.261
Total	310	310	0		0.341

* Significant at 1%

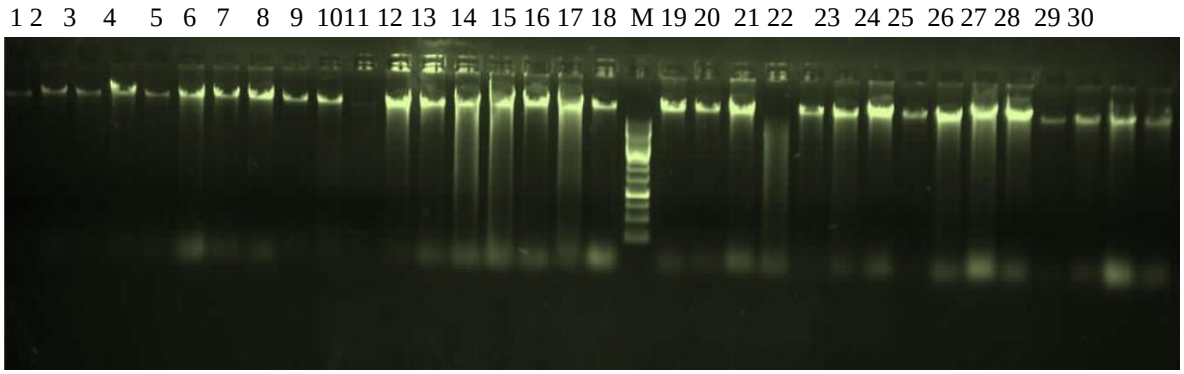

Plate 9: Representative fig. of DNA band pattern of F_2 Plants. (Lane M represents 1kb ladder)

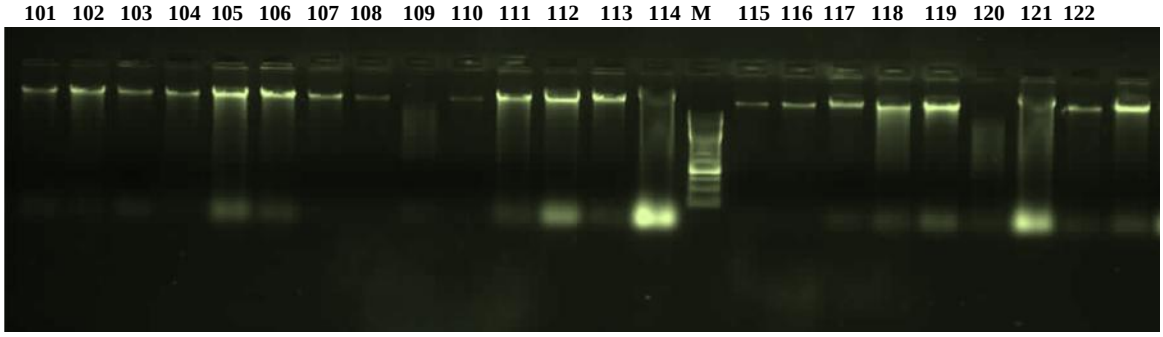

Plate 10: Representative fig. of DNA band pattern of F_2 Plants. (Lane M represents 1kb ladder)

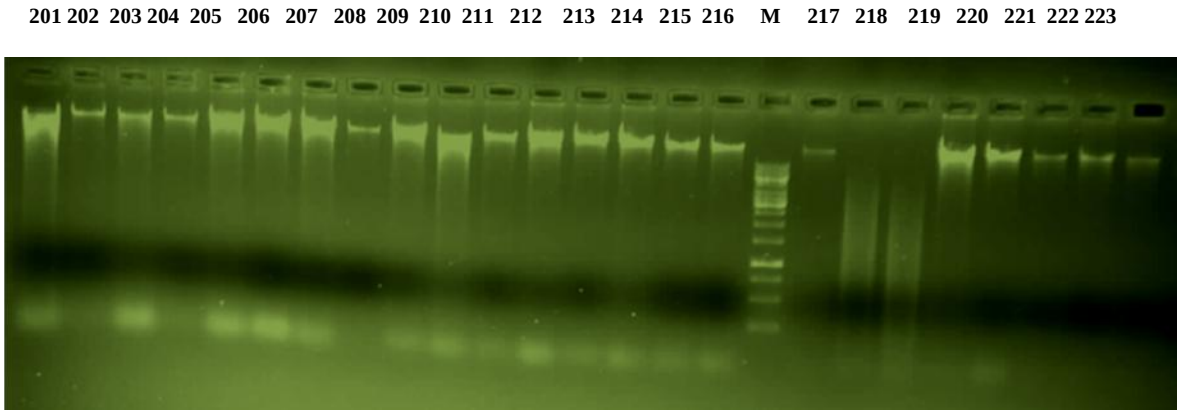

Plate 11: Representative fig. of DNA band pattern of F_2 Plants. (Lane M represents 1kb ladder)

The ratio of resistant plants to susceptible plants was found to be approximately (3:1). This showed that resistance to bacterial wilt in the selected F_2 population was controlled by a single dominant gene.

Ooze-out test was conducted in F_2 plants. Wilt symptom showing plants were selected to confirm whether wilt was due to *Ralstonia* or any other wilt like fusarium wilt or physiological wilt. The root rhizosphere area was cleaned properly to remove soil particles adhering to the plant. A slant cut was made in the root and dipped into a test tube containing clear, distilled water. After 5 mins, milky white streaks of ooze were seen descending down into the water from the cut portion of roots in the plants affected by bacterial wilt (Plate 6). This was the confirmatory test for *Ralstonia* wilt.

4.2. EXPERIMENT 2: Identification of polymorphic SSR markers for Brinjal parents (IIHR-500A and IIHR-575) used in mapping population.

4.2.1 DNA extraction

Optimisation of DNA isolation protocol for brinjal genotypes

In this study, DNA was extracted from the fresh leaf sample of brinjal using CTAB protocol (2%). Brinjal contains more polyphenols which were found to interfere with the isolation of DNA as well as further downstream applications. Therefore, optimization of DNA extraction procedure was undertaken. Increasing the concentration of ß-mercaptoethanol in the extraction buffer by two folds (10µl/ml) gave better results with improved quantity of DNA. The integrity and purity of DNA was checked through 0.8% agarose gel electrophoresis.

DNA was isolated from tender leaves of parental population(10 plants from each) IIHR-500A(P_1) and IIHR-575(P_2). Purity of the DNA was obtained by the ratio of A_{260}/A_{280} by using UV spectrophotometer. A

representative image showing the plant genomic DNA of IIHR-500A, IIHR-575 in 0.8% agarose is shown in plates. The yield of DNA was in the range of 50 µg to over 300 µg per g leaf tissue with the A_{260}/A_{280} ratio ranging from 1.56 to 2.21. The DNA quantification based on UV spectrophotometer was taken into consideration for subsequent EST-SSR, genomic SSR and tomato *Ralstonia* QTL SSR screening. The quality and integrity of DNA was found to be good as per the gel electrophoresis results.

4.2.2.Molecular studies

4.2.2.1.EST-SSR Analysis

For EST-SSR analysis, 39 primers were employed for screening of bulked parental DNA samples i.e., IIHR-500A and IIHR-575. Bulking of DNA was done to detect polymorphic bands in parental population. It was difficult to use the shortlisted primers for 50 individual plants for each of the parental population.Thus bulking of DNA was done following bulk segregant analysis principle (BSA). The selected primers belonged to EEMS series. The amplification pattern of the 39 EST-SSR primers is mentioned in (Table 8)

Out of 39 EST-SSR primers used for screening and detection of polymorphism in bulked parental DNA samples, in 36 primers amplification was seen. At first, 3% agarose gel was used to check polymorphism in the parental DNA samples (Plate 12, 13). No polymorphic bands were visualised as all the bands generated were monomorphic in Agarose gel.

As no polymorphism was detected between parental populations of IIHR-500A and IIHR-575, the EST-SSR primers were not used for further screening of F_2 population.

Table 8. Amplification pattern of EST-SSR primers in bulked IIHR-500A and IIHR-575(parental population)

Sl. No.	EST SSR ID No./ Loci	Remarks
1	EEMS06	Monomorphic
2	EEMS07	Monomorphic
3	EEMS10	Monomorphic
4	EEMS12	Monomorphic
5	EEMS13	Monomorphic
6	EEMS14	Monomorphic
7	EEMS15	Monomorphic
8	EEMS16	No amplification
9	EEMS17	Monomorphic
10	EEMS18	Monomorphic
11	EEMS19	Monomorphic
12	EEMS20	Monomorphic
13	EEMS21	Monomorphic
14	EEMS22	Monomorphic
15	EEMS23	Monomorphic
16	EEMS24	Monomorphic
17	EEMS25	Monomorphic
18	EEMS26	Monomorphic
19	EEMS28	Monomorphic
20	EEMS29	Monomorphic
21	EEMS30	Monomorphic
22	EEMS31	No amplification
23	EEMS32	Monomorphic
24	EEMS33	Monomorphic
25	EEMS34	Monomorphic
26	EEMS35	Monomorphic
27	EEMS36	Monomorphic
28	EEMS37	Monomorphic
29	EEMS38	Monomorphic
30	EEMS39	Monomorphic
31	EEMS41	No amplification
32	EEMS42	Monomorphic
33	EEMS44	Monomorphic
34	EEMS45	Monomorphic
35	EEMS46	Monomorphic
36	EEMS47	Monomorphic
37	EEMS48	Monomorphic
38	EEMS49	Monomorphic
39	EEMS50	Monomorphic

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

S R S R S R S R S R S R S R S R S R S R S R SR SR SR SR SR S R

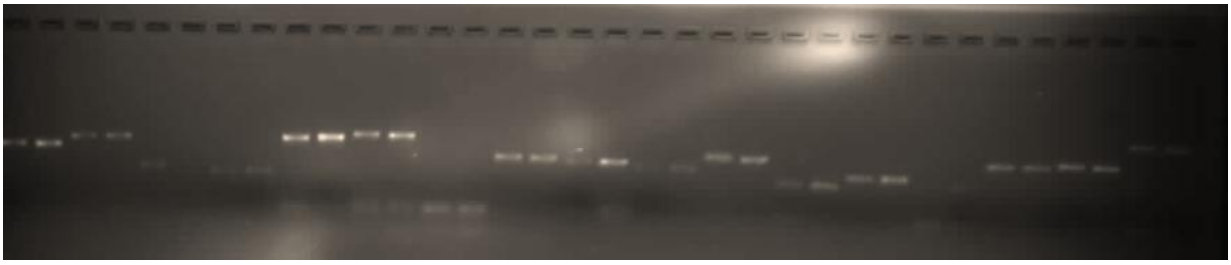

Plate 12: Representative fig. of amplification pattern of 12 EST SSR primers in IIHR-575 (SUSCEPTIBLE,S) and IIHR-500A (RESISTANT,R); 1-17 represents the primers Sl. No. given in (Table 3)

21 22 23 24 25 26 27 28 29

S R S R S R S R S R S R S R S R S R

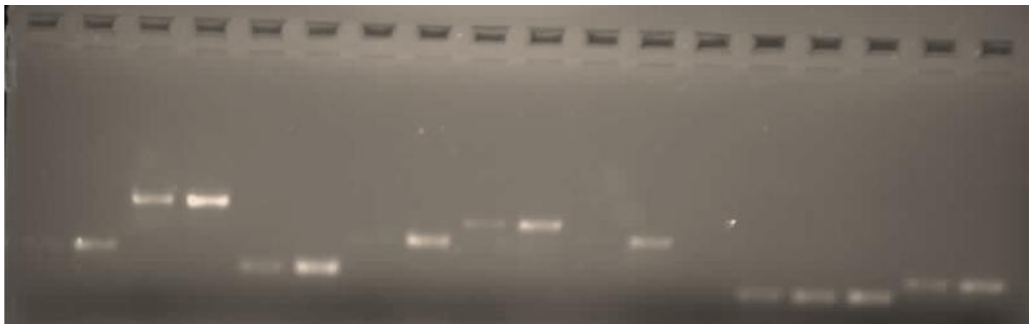

Plate 13: Representative fig. of amplification pattern of 12 EST SSR primers in IIHR-575 (SUSCEPTIBLE,S) and IIHR-500A (RESISTANT,R); 21-29 represents the primers Sl. No. given in (Table 3)

Table 9. Amplification pattern of polymorphic genomic SSR primers in parental population

Sl. No.	Primer Name	Remarks
1	emi04P17	Polymorphic
2	emb01N07	Polymorphic
3	emf21C11	Polymorphic
4	emf21E10	Polymorphic
5	emg11I03	Polymorphic
6	eme03A5	Polymorphic
7	emd01C11	Polymorphic
8	eme01B01	Polymorphic
9	eme36B08	Polymorphic
10	emg21I10	Polymorphic
11	eme01D03	Polymorphic
12	emg11P03	Polymorphic
13	em135 [a]	Polymorphic
14	em119 [a]	Polymorphic
15	emd01C04	Polymorphic

4.2.2.2.Genomic SSR Analysis

Due to non-polymorphism between parental population using 39 EST-SSR primers, genomic SSR primers were used. For genomic SSR analysis, 167 primers were employed for screening of bulked parental DNA samples i.e. IIHR-500A and IIHR-575.

A total of 167 genomic SSR primers were screened for parental bulk DNA samples i.e., IIHR-500A and IIHR-575. Out of 167 primers, 155 primers got amplified. no amplification was seen for 12 primers. From the 155 amplified primers, in 15 primers polymorphism was detected. Polymorphism was decided on the basis of presence or absence of bands as well as difference in position of the bands. Only those primers whish showed clear and distinct bands were only considered to be polymorphic. A representative image of amplification pattern of genomic SSR primers is shown in plates 14 to 19.

4.2.2.3. Tomato *Ralstonia* QTL SSR Analysis:

Tomato *Ralstonia* QTL SSR analysis was done in parental populations i.e. IIHR-500A and IIHR-575 in which a total of 71 Tomato *Ralstonia* QTL SSR primers were used for screening the parents.

A total of 71 Tomato *Ralstonia* QTL SSR primers were screened for parental bulk DNA samples *i.e.,* IIHR-500A and IIHR-575. Out of 71 primers, 46 primers got amplified. no amplification was seen for 25 primers. From the 46 amplified primers, in 6 primers polymorphism was detected. Polymorphism was decided on the basis of presence or absence of bands as well as difference in position of the bands. Only those primers whish showed clear and distinct bands were only considered to be polymorphic. A representative image of amplification pattern of Tomato *Ralstonia* QTL SSR primers is shown in plates 20 and 21.

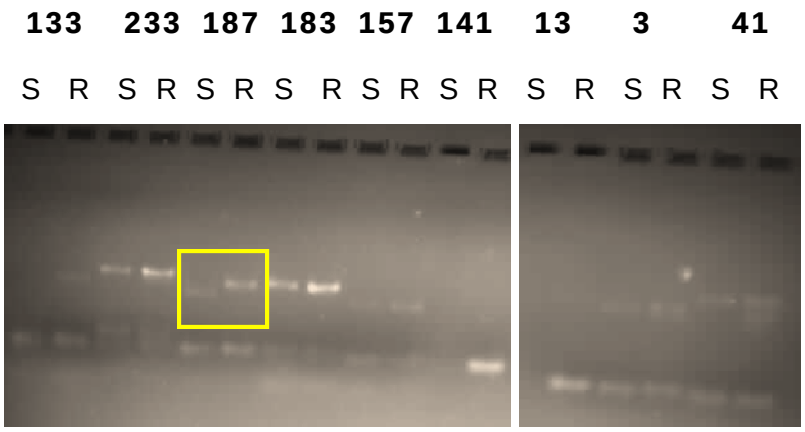

Plate 14: Representative fig. of amplification pattern of 9 Genomic SSR primers in IIHR-575 (SUSCEPTIBLE, S) and IIHR-500A (RESISTANT, R); Sl. No. represents the primer No. given in the (Table 4)

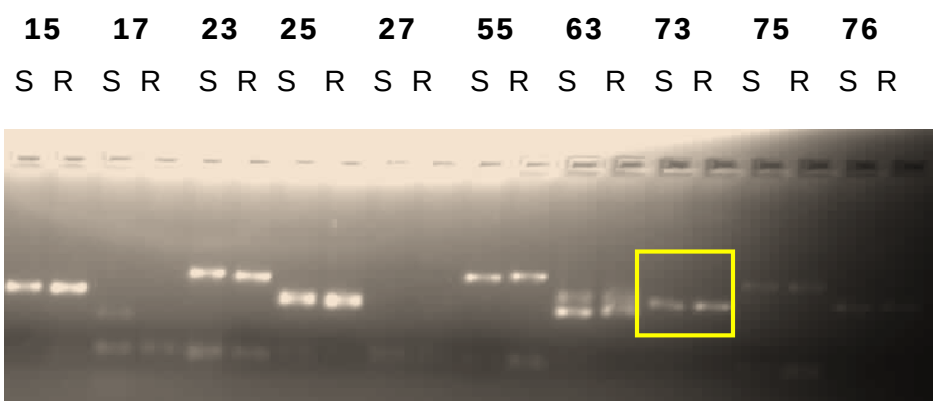

Plate 15: Representative fig. of amplification pattern of 9 Genomic SSR primers in IIHR-575(SUSCEPTIBLE, S) and IIHR-500A (RESISTANT, R); Sl. No. represents the primer No. given in the (Table 4)

77 79 91 97 107 109 125 127 135 143 171 173 189

S R S R S R S R S R S R S R S R S R S R S R S R S R

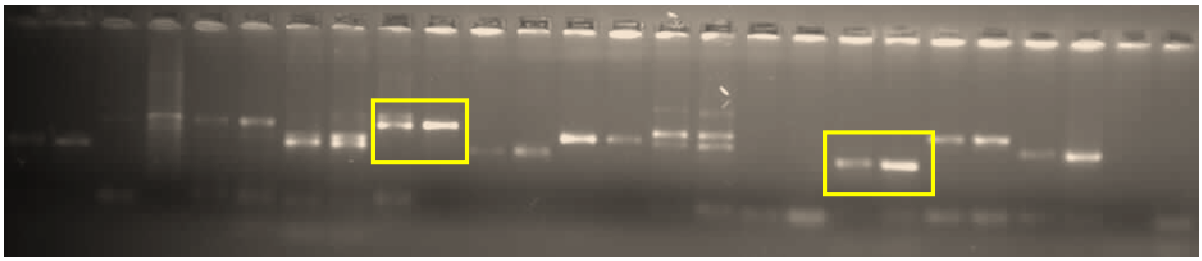

Plate 16: **Representative fig. of amplification pattern of 9 Genomic SSR primers in IIHR-575(SUSCEPTIBLE, S) and IIHR-500A (RESISTANT, R); Sl. No. represents the primer No. given in the (Table 4)**

77 79 91 97 107 109 125 127 135 143 171 173

S R S R S R S R S R S R S R S R S R S R S R S R

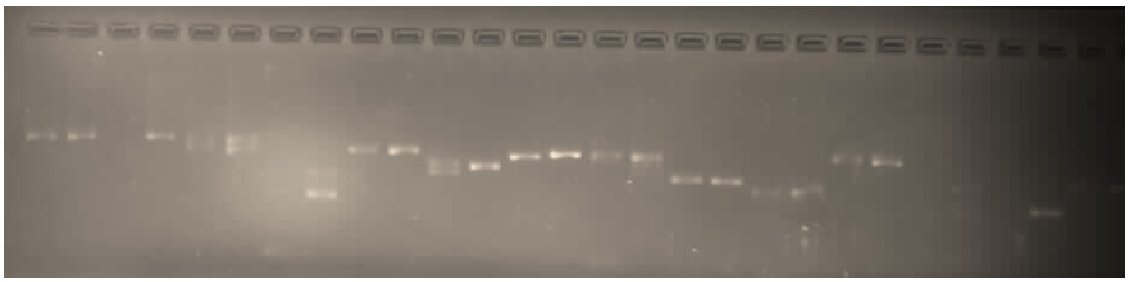

Plate 17: **Representative fig. of amplification pattern of 12 genomic SSR primers in IIHR-575(SUSCEPTIBLE, S) and IIHR-500A (RESISTANT, R); Sl. No. represents the primer No. given in the (Table 4)**

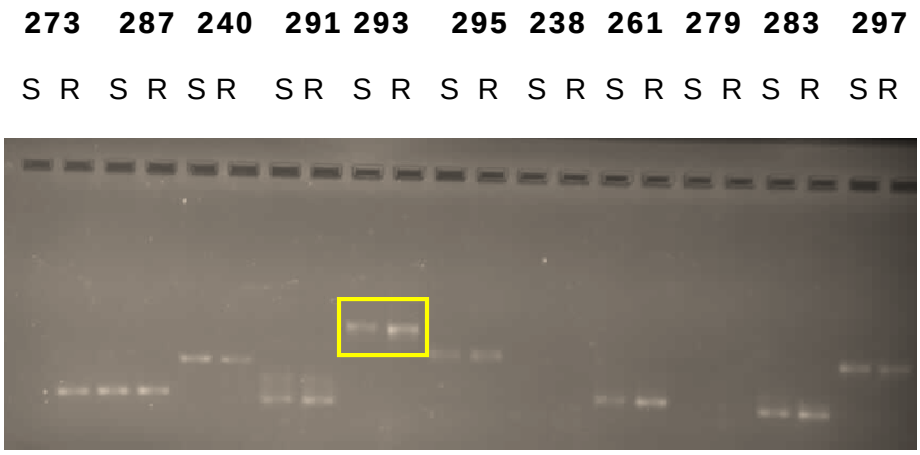

Plate 18 : Representative fig. of amplification pattern of 12 genomic SSR primers in IIHR-575(SUSCEPTIBLE, S) and IIHR-500A (RESISTANT, R); Sl. No. represents the primer No. given in the (Table 4)

Plate 19: Representative fig. of amplification pattern of 8 genomic SSR primers in IIHR-575(SUSCEPTIBLE, S) and IIHR-500A (RESISTANT, R); Sl. No. represents the primer No. given in the (Table 4)

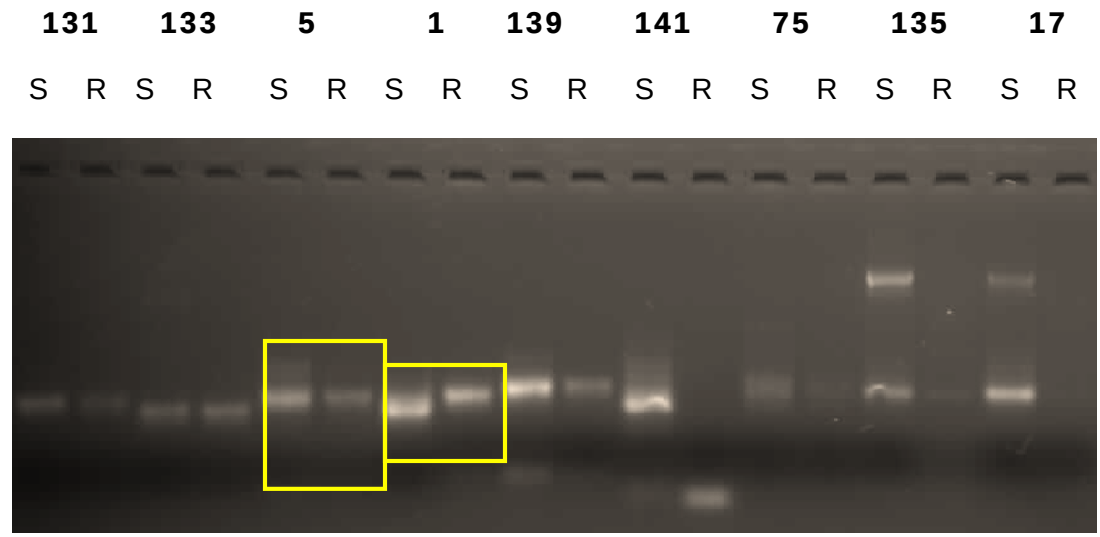

Plate 20: **Representative fig. of amplification pattern of 10 tomato *Ralstonia* QTL SSR primers in IIHR-575(SUSCEPTIBLE, S) and IIHR-500A (RESISTANT, R); Sl. No. represents the primer No. given in the (Table 5)**

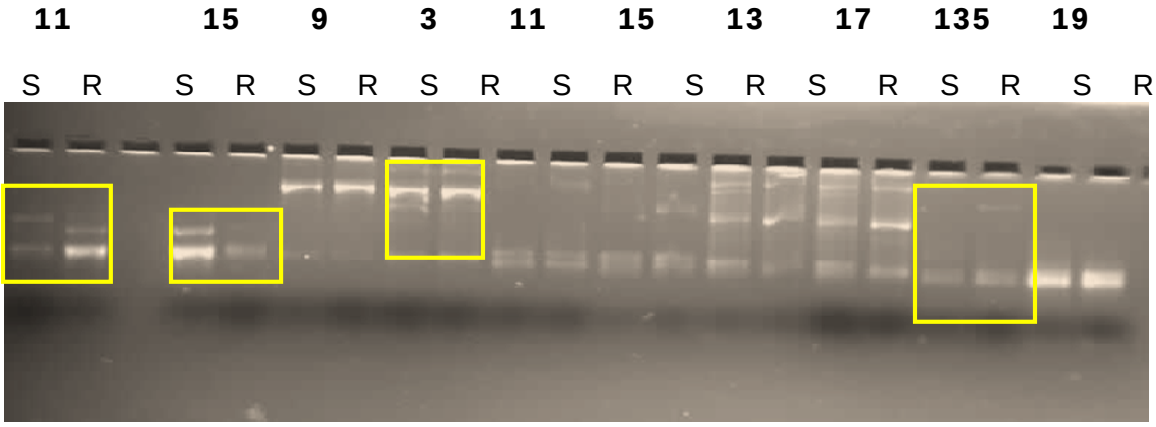

Plate 21: **Representative fig. of amplification pattern of 10 tomato *Ralstonia* QTL SSR primers in IIHR-575(SUSCEPTIBLE, S) and IIHR-500A(RESISTANT, R); Sl. No. represents the primer No. given in the (Table 5)**

4.3 EXPERIMENT 3: Identification of molecular markers(SSRs) linked to bacterial wilt in brinjal

Bulk segregant analysis procedure founded by Michelmore *et al.* (1991), was adopted to prepare bulk resistant DNA from 273 resistant F_2 plants and bulk susceptible DNA from 73 F_2 susceptible plants. The 15 short-listed genomic SSR primers and 6 short-listed Tomato *Ralstonia* QTL found polymorphic in parental population were used to screen the bulk resistant and bulk susceptible from F_2 population. The amplification pattern of the above genomic SSR primers is given in the (table 10) and Tomato *Ralstonia* QTL is given in the (Table 11, 12).

All the 15 polymorphic genomic SSR primers amplified in F_2 population but no polymorphic bands were observed among the bulk resistant and bulk susceptible F_2 population. The bands produced were monomorphic type (Plate 22).

All the 6 polymorphic primers amplified in F_2 population but no polymorphic bands were observed among the bulk resistant and bulk susceptible F_2 population. The bands produced were monomorphic type (Plate 23).

Table 10. Amplification pattern of Tomato *Ralstonia* QTL SSR Primers in bulked IIHR-575 and IIHR-500A (parental population)

Sl. No.	Primer Name	Remarks
1.	emh11G09	Polymorphic
2.	emh01J23	Polymorphic
3.	emh05B12	Polymorphic
4.	emh05B13	Monomorphic
5.	emf01M12	Monomorphic
6.	emg21k22	Monomorphic
7.	emf01P21	Polymorphic
8.	emf01P22	Polymorphic
9.	emd18E02	Monomorphic
10.	emd18E03	No amplification
11.	emd18E04	No amplification
12.	emh01O20	No amplification
13.	emh01O21	No amplification
14.	emh01O22	Monomorphic
15.	emh01O23	Monomorphic
16.	emg11K23	Monomorphic
17.	emg21F05	Monomorphic
18.	emf11N03	Monomorphic
19.	emf11N04	Monomorphic
20.	emf11N05	Monomorphic
21.	emf11N06	No amplification
22.	emd16C09	No amplification
23.	emd16C10	No amplification
24.	emd16C11	No amplification
25.	emf01P24	Monomorphic
26.	emf01P25	Monomorphic
27.	emf01P26	Monomorphic
28.	emf01P27	Monomorphic
29.	emf01P28	No amplification
30.	emf01P29	No amplification
31.	emf01P30	No amplification
32.	emf01P31	No amplification
33.	emd02A05	No amplification
34.	emd02A06	No amplification
35.	emf11A03	No amplification
36.	emf11A04	No amplification
37.	emf01J09	No amplification
38.	emi02F22	No amplification
39.	eme02B08	No amplification
40.	eme02B09	No amplification
41.	emg1I17	Monomorphic
42.	emb01K07	Monomorphic
43.	emb01K08	Monomorphic
44.	emb01K09	Monomorphic
45.	emd17D06	Monomorphic
46.	eme11G05	No amplification
47.	eme11G06	No amplification
48.	emd18B04	No amplification
49.	emd18B05	No amplification
50.	emd13E02a	No amplification
51.	emh03B06	Monomorphic
52.	eme08A04	Monomorphic
53.	eme08A05	Monomorphic
54.	emd05D12	Monomorphic
55.	eme05B09	Monomorphic
56.	eme05B10	Monomorphic
57.	emd07A07	Monomorphic
58.	emd07A08	Monomorphic
59.	emh01J03	Monomorphic
60.	emh01J04	Monomorphic
61.	emb01C12	Monomorphic
62.	emb01C13	Monomorphic
63.	eme12G04	Monomorphic
64.	emh02E08	No amplification
65.	emh11B18	Monomorphic
66.	emh11B19	Monomorphic
67.	eme03B08	Monomorphic
68.	eme01G10	Polymorphic
69.	eme01G11	Monomorphic
70.	eme01G12	Monomorphic
71.	emh01F12	Monomorphic

Table 11. Amplification pattern of genomic SSR primers in F_2 Population

Sl. No.	Primer Name	Type of bands produced
1	emi04P17	Monomorphic
2	emb01N07	Monomorphic
3	emf21C11	Monomorphic
4	emf21E10	Monomorphic
5	emg11I03	Monomorphic
6	eme03A5	Monomorphic
7	emd01C11	Monomorphic
8	eme01B01	Monomorphic
9	eme36B08	Monomorphic
10	emg21I10	Monomorphic
11	eme01D03	Monomorphic
12	emg11P03	Monomorphic
13	em135 [a]	Monomorphic
14	em119 [a]	Monomorphic
15	emd01C04	Monomorphic

Table 12. Amplification pattern of tomato *Ralstonia* QTL SSR primers in F_2 population.

Sl. No.	Primer Name	Type of bands produced
1	emh11G09	Monomorphic
2	emh01J23	Monomorphic
3	emh05B12	Monomorphic
4	emf01P21	Monomorphic
5	emf01P22	Monomorphic
6	eme01G10	Monomorphic

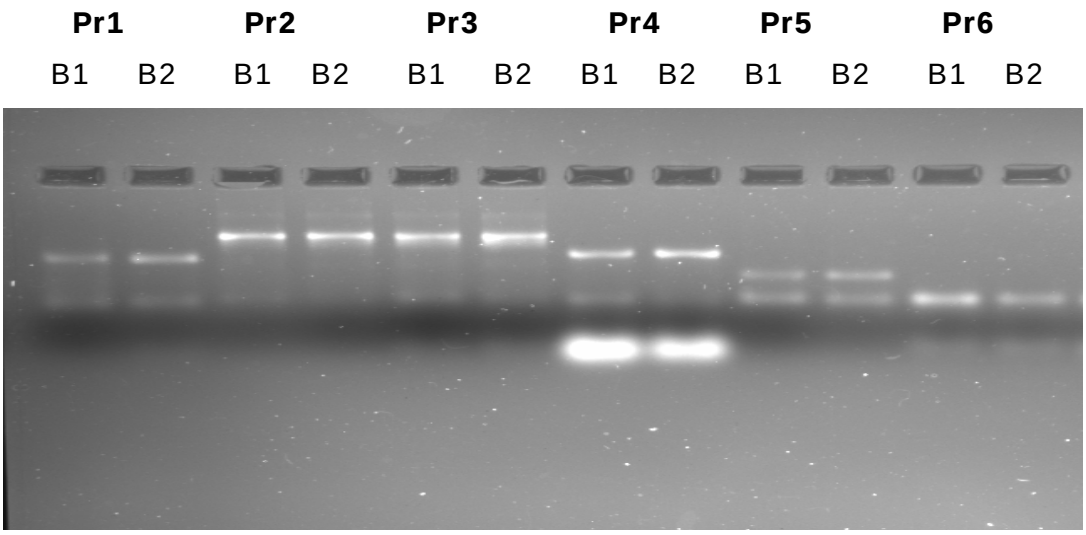

Plate 22: Amplification pattern of genomic SSR primers in IIHR-575(F_2 BULK, B1) and IIHR-500A (F_2 BULK, B2) on 3% agarose gel showing all monomorphic bands, Pr represents the primers used and [Pr 1: emi04H10, Pr 2: emb01D01, Pr 3: emf21K08, Pr 4: emg11M21, Pr 5: emb01J19, Pr 6: emb01F],

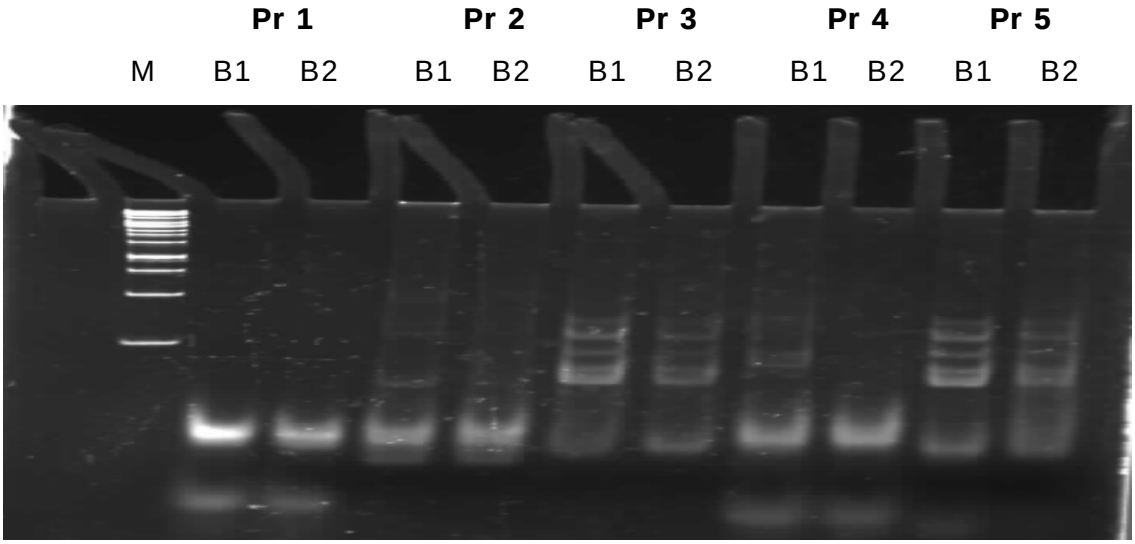

Plate 23: Amplification pattern of Polymorphic tomato *Ralstonia* QTL SSR primers in IIHR-575 (F_2 BULK,B1) and IIHR-500A (F_2 BULK,B2) on 8% PAGE (Lane M represents 1kb ladder) showing all monomorphic bands, Pr represents the primers used and [Pr 1: emh11G09, Pr 2: emh01J23, Pr 3: emh05B12, Pr 4: emf01P21, Pr 5: eme01G10].

DISCUSSION

V. DISCUSSION

Brinjal is the third most important vegetable in the world after potato and tomato in terms of production and consumption. Advances in molecular biology have provided new tools like marker assisted selection (MAS), which increases the efficiency of breeding programmes especially resistance breeding. Although the screening process of the genotypes is complex and time-consuming, MAS simplifies it. With MAS, once the gene of interest is tagged with a molecular marker, selection for that gene can be based on the marker rather than the phenotype of the individual which embodies the gene.

5.1 Phenotyping of brinjal genotypes (IIHR-500A, IIHR-575 and F_2 population) for bacterial wilt resistance

The present study employed an initial screening of two parental genotypes of brinjal known to be having resistance and susceptibility for bacterial wilt. The two parents used in the study were IIHR-500A and IIHR-575 developed in this institute. IIHR-500A was selected for its high resistance to bacterial wilt. But as fruits are not up to commercial standards, it is not recommended for commercial cultivation. The other parent IIHR-575 although produces fruits of good cooking quality, it is highly susceptible to bacterial wilt.

In the parental population respective resistance and susceptibility traits were observed after bacterial (*Ralstonia solanacerum*) inoculation. After phenotyping of the F_2 population was done, the ratio of resistant to susceptible brinjal plants was found to be approximately (3:1) which was proved by chi-square analysis. The calculated value of x^2 from the data in Table 4.1 was 0.341, while that from the x^2 table at 0.05 probability and 1 % degree of freedom was 3.841. Therefore, the null hypothesis was

accepted, and it was concluded that the data were in agreement with 3:1 ratio.

This showed that the resistant trait was controlled by a single dominant gene as per Mendelian genetics (Swaminathan and Srinivasan, 1972). The dominance of resistant trait over the susceptible trait has been studied and proved in previous studies (Akiba, 1972; Vijayagopal and Sethumadhavan, 1973 Gopinath and Madalageri, 1986; Patil, 1998; Feng, *et al.,* 2003; Zhu, *et al.,* 2004; Ajjappalavara, *et al.,* 2008). The result obtained was in accordance with earlier report.

5.2 Identification of polymorphic markers for the parents (IIHR-500A and IIHR-575) used in the mapping population.

Different methods can be employed to identify and develop molecular markers that would contribute to MAS. In the present study, PCR-based approaches of DNA markers were employed to screen resistance of brinjal genotypes for bacterial wilt.

A good quality DNA is essential for reproducible results in molecular profiling that would lead to the identification and development of molecular markers. One important factor is that differences in the template DNA concentrations of two individual DNA samples could result in the loss or gain of some bands (Bardakci, 2001). Initial attempts with DNA extraction from fresh leaves of brinjal resulted in low yields and poor quality of DNA. Brinjal is known to contain inhibitors like pigments and phenols. It required optimizing the extraction procedure with double the normal quality of β- mercaptoethanol, which is a reducing agent, to obtain good quality and yield DNA. The DNA yield ranged from 50 µg to over 300 µg per g leaf tissue and the A260/A280 ratio ranged from 1.56 to 2.21. The resultant DNA appeared to be of adequate quality for the molecular marker studies.

Bulk segregant analysis (BSA) is a rapid procedure for identifying markers in specific regions of the genome (Michelmore *et al.*, 1991, Sestras et al., 2009). This involves screening for differences between two pooled DNA samples derived from a segregating population that originated from a single cross. Within each pool or bulk, the individuals are identical for the trait or gene of interest, but are arbitrary for other genes. Two pools contrasting for a trait (in this case resistance and susceptibility for bacterial wilt) are analyzed to identify the markers that distinguish them.

One of the most time consuming requirements of marker identification programmes is the need to screen entire mapping population with every primer which has been reduced by bulk segregant analysis. Bulk segregant analysis (BSA) was followed for pooling of DNA. DNA was isolated from ten individual plants of two parental genotypes and two pooled samples of each parental genotype were used for screening using EST-SSR, genomic SSR and Tomato *Ralstonia* QTL SSR primers.

For EST-SSR screening, a total of 39 primers were employed in screening out of which all 39 primers produced monomorphic band(s) for IIHR-500A and IIHR-575 genotypes (Plate 12, 13). EST-SSR primers have been reported to be less polymorphic compared with genomic SSRs in crop plants because of greater DNA sequence conservation in transcribed regions (Varshney *et al.*, 2005). EST-SSRs are located within genes, and thus more conserved across species, they may be less polymorphic than anonymous SSRs. (Cho *et al*., 2000, Liewlaksaneeyanawin *et al*., 2004, Chabane *et al.*, 2005 and Pashley *et al*., 2006). Our results again shows low polymorphism with EST-SSR as expected with the previous results reported.

For genomic SSR screening, a total of 167 primers were used. Out of the 167, 15 primers were found to show polymorphism in both IIHR-500A and IIHR-575 on 3% agarose gel (Plate 14 to 19). High degree of allelic variation revealed by microsatellite markers results from variation in number of repeat-motifs at a locus caused by replication slippage and/or unequal crossing-over during meiosis. Polymerase slippage during DNA replication or slipped strand mis-pairing is considered to be the main cause of variation in number of repeat units of a micro satellite resulting in length polymorphisms that can be detected by high percentage of agarose gel (Zietkiewicz *et al.,* 1994). 20.5% of genomic SSRs showed polymorphic as against 0.0% of EST-SSR of primers used. These results again confirmed genomic area shows greater polymorphism as compared to transcribed regions of the genome.

Screening 71 Tomato *Ralstonia* QTL SSR primers for the parental population was performed, out of which 6 were found to be polymorphic (Plate 20, 21).These results again confirmed genomic area shows greater polymorphism as compared to transcribed regions of the genome.

5.3 Identification of molecular markers linked to bacterial wilt in brinjal

The screening approach helped in short listing some promising genomic SSR primers and Tomato *Ralstonia* QTL SSR primers that showed polymorphic PCR amplification products specific to bacterial wilt resistance in parental population. Such short-listed primers were taken up for repeated validation screenings using fresh batch of DNA of F_2 plant population.

The 15 polymorphic genomic SSR primers (emi04P17, emb01N07, emf21C11, emf21E10, emg11I03, eme03A5, emd01C11, eme01B01,

eme36B08, emg21I10, eme01D03, emg11P03, em135 [a], em119 [a] and emd01C04) and 6 polymorphic tomato *Ralstonia* QTL SSR primers (emh11G09, emh01J23, emh05B12, emf01P21, emf01P22 and eme01G10) for the parental population were used for screening of bulk resistant and bulk susceptible DNA of plants of F_2 population. In F_2 population, no polymorphism (plate 22, 23) was observed for resistant and susceptible plants. This may show that the resistance and susceptibility trait may not be linked. Further, for identifying markers linked to this population there may be the need for a larger plant population and additional primers.

Future line of work:

1. Screening for additional genomic SSR primers in a parental population and larger F_2 population of 1000 plants.
2. Screening for polymorphic genomic SSR primers in BC_1 and BC_2 population.
3. Use of other markers like SNP for identification of markers linked to bacterial wilt resistance.
4. Using these selected markers, linkage maps and chromosome maps can be constructed.

SUMMARY

VI. SUMMARY

The present study entitled **"Phenotyping and Identification of molecular markers(SSR) linked to bacterial wilt resistance in Brinjal"** was conducted at the Division of Biotechnology, Indian Institute of Horticultural Research (IIHR), Hessaraghatta, Bangalore during a period of August, 2011 to June, 2012. The main objective of the study was to identify markers that are linked to bacterial wilt resistance in brinjal and screening of wilt resistant brinjal genotypes. PCR-based molecular approaches like EST-SSR markers, genomic SSR and Tomato *Ralstonia* QTL SSR approach were tried towards attaining the above goal. The salient findings have been summarized below.

The two parental lines, IIHR-500A(Resistant) and IIHR-575(susceptible) and its F_2 population developed by the Division of Vegetable crops, IIHR, Hessaraghatta, Bangalore were used in this study. The two parental populations differed for contrasting traits of resistance and susceptibility. In the F_2 population, the resistant plants to susceptible plants ratio was found to be approximately (3:1) which proved that the resistant trait in the population studied was controlled by a single dominant gene.

The primary requirement for the molecular marker studies is good quality DNA. Optimization of DNA extraction protocol was undertaken resulting in good yields of quality DNA from different genotypes of brinjal.

The polymorphic loci screenings were undertaken initially using two parental genotypes i.e. IIHR-500A and IIHR-575 DNA. Screening was carried out by using 39 EST-SSR primers, out of 39 EST-SSR primers, all were monomorphic and a set of 167 genomic SSR primers, out of which 15 have been identified as polymorphic. A batch of 71 Tomato *Ralstonia* QTL SSR primers was also tested of which 5 were found to be

polymorphic. The 15 short-listed polymorphic genomic SSR primers and 5 Tomato *Ralstonia* QTL SSR primers were screened in F_2 population but the marker linked to bacterial wilt could not be identified.

The results brought about the following conclusions:

1. Bulk segregant analysis is an efficient tool for reducing the time consumed and easing the process of screening the entire mapping population with every primer.
2. Genotyping of the individual is the very basic screening for the future works like QTL, linkage maps analysis, genome mapping.
3. The SSR markers or microsatellite used for the screening of assigning a molecular / DNA marker may be a time consuming effort. Yet, there lies a greater possibility of finding a unique polymorphic band.

From this it is concluded in this study that, large number of primers are required to screen the F_2 population. Hence development of large number of SSR primer should be targeted before taking up the identification of marker work.

The other approach like candidate gene also may be used by searching the sequence of the genes resistant to *Ralstonia* in other crops.

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APPENDIX

APPENDIX

Reagents for DNA isolation

Extraction buffer: 20 mM NaEDTA, 100 mM Tris Buffer and 1.4 M NaCl were prepared, mixed and pH was adjusted to 8.0. **2% w/v CTAB** (Cetyl Tri Methyl Ammonium Bromide) was prepared by heating CTAB to 60 C to dissolve it and mixed. After autoclaving it was stored at 37 C. 0.2% -mercaptoethanol was added just before use.

For volume of 1 litre extraction buffer preparation, chemicals required were:

Chemicals	Quantity(g)
Na EDTA	7.44
Tris Buffer	13.23
NaCl	81.816
CTAB	20.00

All the above were dissolved in distilled water and the volume made upto 1litre.

1. **Chloroform : Iso-amyl Alchohol :** (24:1) v/v
2. **5M NaCl :** NaCl of weight 292g of was dissolved in 1litre water and autoclaved.
3. **TE Buffer:** 10 mM Tris buffer and 1mM NaEDTA were prepared. From this, for preparing 1 litre TE buffer, 10ml of 10mM Tris buffer and 2ml of 1mM NaEDTA were taken and mixed. pH was adjusted to 8.0 and autoclaved.
4. **7.5M Ammonium Acetate:** 578.1g of Ammonium Acetate was dissolved in 1 litre distilled water and the pH was adjusted to 7.7.

5. **Ethanol:** 76% v/v (76ml absolute ethanol + 24ml distilled water) chilled.
6. **Ethanol:** 95% stored at -20°C.
7. **Absolute alcohol:** Stored at -20°C.
8. **RNAase:** [10mg/ml]; RNAase was dissolved in 10 mM TRIS HCl + 15mM NaCl, pH 7.5, boiled for 5 mins & cooled to room temperature.
9. **Poly Vinyl Poly Pyrrolidone** Powder **(PVPP):**
10. **TAE [Tris Acetate Ethylene Diamine Tetra Acetic Acid (EDTA)] Buffer**: The following chemicals were dissolved in 1 litre of water to prepare 50X TAE and autoclaved.

Reagents	Quantity
Tris base	242.0 g
Glacial acetic acid	57.1 ml of
0.5M EDTA(pH 7.0)	100 ml

11. **Working solution:** 1 X [diluted stock 10 times] was used in case of gel preparation and 0.5 X in case of running buffer.
12. **Bromo phenol blue:** *Stock solution:* 0.25% in 50% glycerol*Working solution:* 200 l stock + 200 l of 50% glycerol
13. **Ethidium bromide:** 10 mg/ml
14. **Agarose powder**: As per the gel strength (%): (0.8 – 2) %

Polyacrylamide Gel Electrophoresis (PAGE)

i) Reagents:

1. Acrylamide stock solution (30%):

   30 g - Acrylamide

   0.8 g – bis-acrylamide

   Volume made up to 100 ml

2. TAE Buffer (50X)
3. Ammonium Persulphate (APS) [10%]: By dissolving 100mg APS in 1ml of distilled water
4. Bromo phenol blue: *Stock solution:* 0.25% in 50% glycerol

   (*Working solution:* 200µl stock + 200µl of 50 glycerol)

Polyacrylamide stock solution of 30 per cent was prepared in advance and stored at 4°C.

Table4. Composition of 8% PAGE (20ml)

Reagents	Volume
Acrylamide (30%)	5.33ml
TAE (50X)	0.4ml
TEMED	33.4µl
APS	83.33µl
Distilled Water	14.06ml

ii) Plate preparation

The glass plates should be thoroughly cleaned before pouring polyacrylamide gel.

Solutions:

1. Bind silane stock solution: 40 ml of 100 per cent ethanol + 150 µl of bind silane.

2. Bind silane working solution: 10 ml stock bind solution + 1ml 10 per cent acetic acid.

3. Repel coat

Protocol:

1. Both the plates are cleaned using detergent. The plates are then rinsed with millipore water and dried with paper towels.
2. The working side of the large plate is wiped with acetone and alcohol and treated with 1000 µl of repel by spreading with paper towels in even sweeps across the whole plate. An ethanol wipe is given after 5 minutes to spread evenly and final polish is given with second ethanol wipe.
3. The small plate is treated twice with bind silane by spreading 500µl of working solution evenly over the whole surface.
4. Spacers are cleaned with ethanol and aligned on the edges of large plate. The smaller plate is placed on top and taped on both sides to secure both plates together.

iii) Pouring of Poly Acrylamide Gel

1. Plates are fixed in the PAGE unit tightly to prevent any gel leakage
2. Polyacrylamide mix (Acrylamide solution+ TAE + TEMED + APS + Distilled Water) is poured slowly between the plates.
3. The comb is placed in reverse direction between plates and the gel will be allowed to polymerize for 1.5 hours.
4. The PAGE unit is transferred to electrophoresis tank containing 0.5X TAE buffer.

5. The comb is removed by sliding it out horizontally.
6. The amplified PCR product (10µl+ 3µl Bromophenol blue dye) is loaded into the wells followed by loading of 5 µl of 100bp marker in the last well.
7. Electrophoresis is done at 100V for about 60-90 min., later increased to 150V till the completion.
8. The plates are to be removed and the gel is transferred into staining tank for Ethidium bromide staining.

iv) Ethidium bromide Staining

1. The gel plates are placed in staining trays (gel facing up): one corner of the backing plate is lifted and the backed gel is peeled away from the rear glass plate. Gels are to be handled with care; they should not be touched with bare hands on the surface until image is taken
2. Ethidium bromide solution (30µl in 500ml distilled water) is to be poured into staining tank and covered immediately to prevent exposure to light.
3. It is to be kept for 20 mins staining with intermittent shaking.
4. The water is drained out.
5. Rinsing to be done with distilled water for 5-20 sec.
6. Gel can be viewed gel documentation system and photographed.