MOLECULAR CHARACTERISATION OF *Ralstonia solanacearum* (Smith) YABUUCHI *et al.* CAUSING BACTERIAL WILT IN SOLANACEOUS VEGETABLES

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Molecular characterisation of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* causing bacterial wilt in solanaceous vegetables"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled **"Molecular characterisation of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* causing bacterial wilt in solanaceous vegetables"** is a record of research work done independently by **Miss Deepa James** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

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DEEPA JAMES

Dedicated to
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Introduction

1. INTRODUCTION

Vegetables constitute an important item of human diet since they form valuable sources of carbohydrates, proteins, vitamins and minerals. The daily minimum requirement of vegetables is 284 g per head, i.e., about 20 per cent of the daily requirement of the total food of an adult. The present production and consumption of vegetables in the country are highly inadequate, being only about one-fourth to one-third of the requirement.

Vegetable crops are broadly classified into thirteen groups, of which the solanaceous fruits viz., tomato, brinjal and chilli belonging to the family Solanaceae, constitute an important group. Tomato is an important "protective food" both because of its special nutritive value and also because of its widespread production. The outstanding value of tomato is due to the presence of special nutrients needed in the diet like vitamin C, vitamin A and different minerals. Brinjal, also known as eggplant, has got nutritional and medicinal properties. It contains vitamin A, vitamin C, Thiamine, Riboflavin and minerals, the content variable in different varieties. Chilli is a valuable crop used as vegetable, spice and condiment and in the preparation of sauces and pickles. They are rich in vitamins, especially A and C. The pungency in chillies is due to an alkaloid, capsaicin, which has good export possibilities.

Solanaceous vegetables are affected by a number of diseases like leaf spots, mosaic, leaf curl, fruit rot, bacterial wilt etc. Among these, the bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al* (=*Pseudomonas solanacearum* E.F.Smith) is the most devastating disease in tropics and subtropics. Since the pathogen is systemic, control is difficult and affected plants completely wilt. The pathogen, being a soil borne bacteria, can spread very quickly, causing yield losses up to 100 per cent.

The earliest report on *P. solanacearum* as the causal organism of bacterial wilt of plants dates back to the end of 19th century (Smith, 1896). In many areas of the world, the disease first appeared and intensified causing major crop losses and migration of human population to other areas. This is particularly true in the once prosperous tobacco growing region Granville in North Carolina, where hundreds of families had to shift to other areas realizing that the tobacco crop on which they had become dependent could not be grown owing to bacterial wilt.

A comprehensive listing of hosts of *R. solanacearum* includes species in fifty plant families with most genera in family Solanaceae. The number of new host species continues to increase. This disease forms a major constraint for vegetable growers specially tomato farmers in lowland and upland tropics where the estimated yield losses range from 15 to 95 per cent.

Effective detection of *R. solanacearum* in soil as well as in plant parts are required for its management by screening resistant lines from the segregating populations. The diagnostic test should be rapid, specific and non-destructive. Earlier, characterisation of bacteria was done mainly based on colony morphology. Fluidal, irregular and mucoid colonies on Kelman's triphenyl tetrazolium chloride (TZC) medium were considered to be virulent and butyrous, smaller round shaped colonies as avirulent. However, some soil bacteria having similar colony morphology as that of *R. solanacearum* can also grow on the medium. This paved way for the development of a series of biochemical tests in order to detect *R. solanacearum*. These identification methods are to be carried out using purified single colonies, which requires more than two weeks before species identification is possible. However, these biochemical tests are quite laborious and time consuming. Recently, molecular techniques have been developed which provide the most compelling evidence. Molecular methods like PCR, RAPD and RFLP are quicker and more sensitive than conventional diagnostic approaches which are based on cultivation of the organism followed by testing of cultural and physiological characteristics. If suitable genetic fingerprints could be developed based on molecular markers, the long and tedious

process of culturing the bacterium and conducting routine microbiological tests could be overcome. Genetic fingerprints can be used for rapid identification of races and biovars in soil, which will ultimately help in taking prophylactic measures against the disease. This will also help the plant breeders for evaluating the resistance of different varieties of plants to different bacterial isolates/races/biovars of *R. solanacearum.*

In view of these, the following aspects are taken up for the present study.

- Isolation of the pathogen from bacterial wilt affected solanaceous vegetables from three different locations viz., Vellanikkara, Kumarakom and Ambalavayal representing three agro climatic zones of Kerala.
- Estimation of aggressiveness and virulence of the isolates.
- Cultural and biochemical characterisation of the isolates.
- Molecular characterisation of isolates using RAPD and RFLP.
- Role of plasmid in exopolysaccharide (EPS) production.

Review of Literature

2. REVIEW OF LITERATURE

Bacterial wilt was first reported from Italy in 1882(Walker, 1952). Burril (1890) made a report of bacterial wilt in connection with an unidentified bacterial disease of potato in USA. Smith (1896) first described the disease and its causal agent and reported its occurrence in potato, tomato and brinjal. Later the disease appeared also in South Africa in 1914 on potato. (Doidge, 1914). Subsequently it was reported to attack chilli, peanut, tobacco, ginger, banana, cucurbits, groundnut, loofah etc. More than 200 species of plants belonging to 33 families with largest number of hosts in Solanaceae were known to be hosts of the bacteria (Kelman, 1953).

E.F. Smith described the bacterium as *Pseudomonas solanacearum.* Later Yabuuchi *et al.* (1992) transferred several species of the rRNA homology group II Pseudomonads, including *Pseudomonas solanacearum* to the genus *Burkholderia.* Sequencing of the 16S rRNA genes and polyphasic taxonomy revealed dichotomy among the species included in the genus *Burkholderia.* This phytogenetic dichotomy had led to the proposal of the new genus, *Ralstonia* (Yabuuchi *et al.*, 1995).

The bacteria is a fastidious soil borne pathogen known for its endemic persistence in soil. The disease occurs in diverse soil types of acidic and alkaline nature. The destructiveness of the disease is due to wide host range of causal organism.

In India, the occurrence of bacterial wilt of tomato was first reported by Hedayathullah and Saha (1941) from West Bengal. Das and Chattopadhyay (1955) made a detailed study of bacterial wilt of brinjal in India. According to them, the average reduction in yield due to the disease was 54.6 to 62.3 per cent. Indian Council of Agricultural Research (1969) made the first report on bacterial wilt of chilli in India from Madhya Pradesh.

2.1 Isolation and maintenance of the pathogen

2.1.1 Isolation of *Ralstonia solanacearum*

There are many chemically defined media suitable for the isolation of bacteria. The media are defined based on their nutritional requirements. In order to differentiate various kinds of bacteria, certain reagents are incorporated into the culture media, thus forming a differential medium. Generally, bacteria are isolated on nutrient agar medium. Earlier, this medium was used to isolate *R. solanacearum* also. Later, several scientists started working for the development of differential media to facilitate the recognition and differentiation of this bacteria from others.

Kelman (1954) developed a medium for the isolation and detection of *P. solanacearum*. Mutants of *P. solanacearum* differing in colony morphology from the normal or wild type were detected readily when the bacterial suspension was streaked on this medium containing triphenyl tetrazolium chloride and examined with obliquely transmitted light. The normal type formed an irregularly round, fluidal, white colony with a pink centre while the mutant formed a round, butyrous, deep red colony with a narrow bluish border. Several other workers also isolated this pathogen from the different sources on the same TZC medium (Husain and Kelman, 1958; Khan *et al.*, 1979; Nayar, 1982; He *et al.*, 1983; Swanepoel and Young, 1988; Prior and Steva, 1990; Paul, 1998).

Later, many selective media were developed for the isolation of *P. solanacearum* from soil. The TZC formed the basic component of most of these media. Karganilla and Buddenhagen (1972) developed a selective medium SM, consisting of a simplified basal medium plus antimicrobial compounds. The colony counts of *P. solanacearum* on this media were reduced by 22 to 33 per cent as compared to a 65 to 99 per cent reduction of other soil bacteria. The antimicrobial compounds used in this medium were inhibitory to certain strains and the recovery of these strains could be improved by amending TZC medium with the same antimicrobial compounds but the colonies of these strains turned

atypical. A new selective medium was then developed to monitor the populations of *P. solanacearum* in naturally and artificially infested soils (Nesmith and Jenkins, 1979). The selective medium was prepared by adding antimicrobial compounds at the time of use. The fluidal colonies of most strains of *P. solanacearum* tested on the selective medium were similar in appearance to those observed on TZC medium. Though most strains grew well on the selective medium, some were partially or completely inhibited. Chen and Echandi (1982) developed another selective medium, TZCCP by adding chloramphenicol (10µg/ml) to the TZC agar, being the basal medium. For the isolation of *P. solanacearum* from soil, this medium reduced the bacterial and fungal contaminants 75 to 95 per cent more than TZC did. But the detection of *P. solanacearum* with this medium was not satisfactory since the bacteria and fungi occasionally contaminated the plates before the appearance of typical colonies of *P. solanacearum* as it required 24 h more incubation at 30°C than on TZC medium. SM-1 and SM-2, the modification of SM-1 were developed by Granada and Sequeira (1983) to isolate *P. solanacearum* from artificially and naturally infested soils. They added crystal violet, thimerosal, polymyxin B sulfate, tyrothricin and Chloromycetin into the basal TZC medium. Here the plating efficiency was excellent, ranging from 80 to more than 100 per cent of that on TZC medium and most strains of *P. solanacearum* grew well on it. Engelbrecht (1994) compared a modified form of a selective medium developed by Graham Lloyd (SMSA) with the original medium (GL) and with the SM-1 medium for isolation of *P. solanacearum* from soil. He found that the plating efficiency on GL was found better than on the other two media, but it did not reduce soil saprobes as well and the recovery rates varied from 36 to 99 per cent. Hara *et al.* (1995) developed the improved Hara and Ono's medium (HOM) named PCCG, which showed higher selectivity of *R. solanacearum*. This PCCG medium was later used for the detection of viable cells of *R. solanacearum*. It was easy to distinguish the fluidal colonies from the butyrous colonies on PCCG medium (Ito *et al.*, 1998).

However, due to the presence of complex strains and wide variability among this bacteria, it is very difficult to develop a medium, which gives the cent percent recovery of *R. solanacearum*.

2.1.2 Maintenance of *Ralstonia solanacearum*

Bacterial cultures can be maintained by different methods like lyophilisation/freeze drying, overlaying the cultures with mineral oil and storing the culture in sterile water without losing the virulence of the bacteria.

Several workers tried to maintain the viability of bacteria by covering the bacterial cultures on solid media with a layer of sterile mineral oil. The pathogenicity of the isolates remained relatively stable as the complete coverage with oil prevented dehydration of the agar and hence the cultures could remain viable for about four years (Kelman and Jenson, 1951; Winstead and Kelman, 1952). For long-term storage, He *et al.* (1983) lyophilized the cultures. But this method is highly expensive.

The stock cultures of different strains of *P. solanacearum* were also prepared by suspending five loops of bacterial growth in 5 ml of sterile distilled water in screw capped test tubes and storing them at 25°C (Husain and Kelman, 1958). Attempts have been made to maintain the viability in a similar way by placing three to five loopfuls of bacterial culture into capped test tubes with 5 ml of sterile distilled water and storing at a temperature of 22°C. This could maintain the cultures relatively free of avirulent mutant types for about 18 to 24 months (Kelman and Person, 1961; Khan *et al.*, 1979; Granada and Sequeira, 1983; Swanepoel and Young, 1988). Stock cultures made in sterile distilled water in screw-capped tubes were also stored at room temperature (He *et al.*, 1983; Prior and Steva, 1990; Mathew *et al.*, 2000). Kumar *et al.* (1993) preserved and stored the cultures of *P. solanacearum* in sterile distilled water in glass vials under refrigerated conditions.

2.2 Pathogenicity

Pathogenicity is the ability of a pathogen to cause disease under a given set of environment while pathogenesis is the chain of events that lead to the development of disease in the host. Certain abnormalities may occur in plants as a result of host pathogen interaction. So pathogenicity can be further explained as the capacity of the pathogen to induce malfunctions or interfere with the physiological activities of the plant.

Pathogenicity of *R. solanacearum* has been studied in relation to the colony characters, methods of inoculation, cross-infectivity and the genetic basis.

2.2.1 Relationship of colony characters with pathogenicity

Okabe (1949) made a special reference to the pathogenicity of colony variants of *P. solanacearum* in Japan. He noted that weakly pathogenic or avirulent mutants differed in colony morphology from the fluidal wild type. However, all fluidal colonies were not highly pathogenic. The virulence was found varying among the morphologically similar colonies derived from a single virulent fluidal colony.

Later, Kelman (1954) correlated pathogenicity with colony characters on tetrazolium medium. Cultures derived from single mutant colonies showing a dark-red pigmentation of tetrazolium medium were either weakly virulent or avirulent. In contrast, cultures from wild type colonies, which remained relatively free of colouration during initial 36 h of growth, were highly pathogenic.

2.2.2 Methods of inoculation

Several inoculation techniques have been tried by many workers for testing pathogenicity and for evaluating resistance to *R. solanacearum*.

Winstead and Kelman (1952) attempted to standardize inoculation technique to test the pathogenicity of different strains of *P. solanacearum* on

respective hosts. They aimed a procedure that would ensure uniform and rapid development of bacterial wilt. For this, they tried (1) stem puncturing at third node below apex, (2) cutting the lateral roots along one side and pouring bacterial suspension over soil, (4) pouring bacterial suspension over soil without injury to roots, (5) dipping roots in a bacterial suspension. Symptoms of wilt became first evident in those plants inoculated by stem puncture at third node below the apex. At the end of 15 days, all plants inoculated either by stem puncture or root injury or root dipping techniques were dead. Inoculation by pouring the bacterial suspension over soil without injuring the roots was not an effective procedure. Although stem inoculations consistently produced higher wilt scores/indices in resistant plants, greatest differentiation between resistant and susceptible plants was obtained by root inoculation.

Kelman (1954) tested the pathogenicity of bacterial cultures by inoculating tomato plants by stem puncture method. All cultures produced rapid wilting and at the end of 14 days, all inoculated plants were severely wilted.

Using stem puncture by means of a hypodermic syringe on eight-week-old tomato plants, Husain and Kelman (1958) noticed intermediate to advanced symptoms of wilting after 10 days of inoculation. In order to assess the varying degrees of virulence for precise dosage of inoculum, Bora and Addy (1982) stem inoculated *P. solanacearum* using a microlitre syringe, which was considered to be an improvement over that of Winstead and Kelman (1952). Stem puncture was also done to test the pathogenicity of strains of *P. solanacearum* from China (He *et al.*, 1983) and from French West Indies (Prior and Steva, 1990). Wang (1971) and AVRDC (1997) used leaf clipping for artificial inoculation in tomato. Root inoculation also had been tested on different hosts and found effective (Khan *et al.*, 1979; He *et al.*, 1983; Swanepoel and Young, 1988 and Paul, 1998).

2.2.3 Cross infectivity

Cross infectivity is the capability of a pathogen isolated from a particular host to infect other plants belonging to the same or different family. This capacity of the pathogen may vary with different strains, races or biovars.

Cross inoculation tests with *R. solanacearum* were carried out among the different solanaceous vegetables. Digat and Escudie (1967) demonstrated that the pathogen isolated from tomato, brinjal and chilli was virulent on solanaceous members. However, in another experiment conducted by He *et al.* (1983), cross inoculation of bacterial strains obtained from China was carried out on six major hosts, viz., brinjal, tomato, capsicum, potato, peanut and tobacco. Rapid wilting was noticed on eggplants on inoculation of all strains. Tomato was susceptible to all strains except that obtained from mulberry. Potato was susceptible to most strains, but the rapidity of wilting varied. Capsicum, peanut and tobacco showed marked differences in their reactions to specific strains but were resistant to the vast majority of strains.

Pathogenicity of South African strains of *P. solanacearum* was tested on potato, tomato, tomato, tobacco, eggplant, capsicum, peanut, sunflower and large thorn apple. Strains obtained from potato designated as biotype II showed no virulence to sunflower, tobacco and peanut, but were virulent to other hosts. Only one strain of biotype II showed virulence to thorn apple. Biotype III strains obtained from tomato and tobacco were virulent to all the eight hosts (Swanepoel and Young, 1988). Madalageri *et al.* (1988) conducted similar experiments with 25 strains of *P. solanacearum* on tomato, capsicum and brinjal. Only three strains were highly virulent on these solanaceous crops. Tomato and brinjal plants were killed while capsicum pants were severely dwarfed.

Strains of *P. solanacearum* were classified on the basis of cross infectivity and host reaction on tomato, brinjal, potato, pepper, peanut, bean and tobacco (Prior and Steva, 1990). Most strains were highly pathogenic on potato,

tomato and brinjal. The strains differed in capacity to cause disease on pepper, peanut, bean and tobacco. All the strains were divided into five groups:

Group 1	-	Pathogenic on all hosts
Group 2	-	Strains pathogenic on pepper
Group 3	-	Strains not pathogenic on pepper or peanut
Group 4	-	A slightly pathogenic strain from eggplant
Group 5	-	Strains not pathogenic on peanut or tobacco

Cross infectivity tests have been conducted in India also by Patel *et al.* (1952) and Hingorani (1956). They reported that the potato isolate could not infect tobacco and chilli plants. Das and Chattopadhyay (1955) and Chattopadhyay and Mukherjii (1969) reported that chilli could be one of the hosts for *P. solanacearum*. Devi (1978) observed that chilli strain of *P.solanacearum* caused high degree of wilting in tomato and brinjal, and that the brinjal and tomato isolates were capable of cross infecting each other. However, chilli isolate failed to produce disease on tobacco or potato whereas brinjal and tomato could be one of the hosts of the pathogen (Khan *et al.*, 1979; Jyothi, 1992). Isolates from tomato, brinjal and chilli were cross inoculable, but produced symptoms with varying intensities (Paul, 1998).

2.2.4 Genetic basis of pathogenicity

Recent reports indicate possibility of genetic approach for studying the pathogenicity in the plant pathogenic bacteria.

Boucher *et al.* (1987) reported the existence of *hrp* (*h*ypersensitivity *r*esponse and *p*athogenicity) genes in the bacteria, which were required for the initiation of disease symptoms on host plants. By the use of Tn5 random mutagenesis, they could isolate 13 mutants, which had lost pathogenicity towards their homologous host, tomato due to the mutation at *hrp* genes. Based on the studies in tobacco, it was confirmed that the pathogenicity of *P.solanacearum*

depends on *hrp* genes, which govern the secretion of proteins mediating host/bacteria interactions (Arlat and Boucher, 1989; Boucher, 1998).

Huang *et al.* (1990) reported a second cluster of genes viz., *dsp* genes specifying pathogenicity in *P. solanacearum*. Insertion of Tn5-lac in the newly identified genes resulted in the loss of pathogenicity.

More details of *hrp* genes will be covered under 2.4.3 in hypersensitivity test

2.3 Aggressiveness and virulence

Virulence can be defined as the relative ability of the pathogens to cause disease. The term 'aggressiveness' is often used to describe the capacity of a pathogen to invade and grow in its host plant and to reproduce on or in it. Both virulence and aggressiveness can be used to measure the pathogenicity of various isolates of the pathogen.

Virulence of different *R. solanacearum* strains were compared usually using a disease rating scale. Winstead and Kelman (1952) compared the virulence of three strains of *P. solanacearum* on a susceptible cultivar of tomato, L390 using the following rating scale.

0 - no symptoms

1 - 1 leaf partially wilted

2 - 2 or 3 leaves wilted

3 - all except the top 2 or 3 leaves wilted

4 - all leaves wilted

5 - plant dead

Nielson and Haynes (1960) evaluated the virulence of different strains of *P. solanacearum* on tomato by giving the grades from 1 to 5 where, 1 – no symptoms, 2 – 1/3 of leaves wilted, 3 – 2/3 of leaves wilted, 4 – whole plant

wilted, 5 – dead plant. These data were then used to calculate the bacterial wilt index in order to compare the virulence.

Quantitative aspects were used for assessing virulence of *P.solanacearum* isolates belonging to race 1, biovar 3 in tomato plants (Bora and Addy, 1982). Six isolates when inoculated with four dosages viz., 10^3, 10^4, 10^5 and 10^6 CFU/ml into tomato stem tissues, the responses varied for the isolates in respect of the development of wilting symptoms for a particular dose of inoculum. The highest dose of 10^6 CFU/ml containing about 2000 viable cells produced the earliest wilting. A comparison of 50 per cent response in symptom expression for the different doses for all the isolates clearly demonstrated the differences in virulence amongst the isolates.

A new method to assess virulence of *P. solanacearum* in tomato was developed in Nepal (Adhikari, 1993). Aggressiveness of the bacteria was measured in terms of incubation period (IP), i.e., the number of days before plant developed visible symptoms, and latent period (LP_{50}), i.e., the number of days before wilting reached 50 per cent.

Silveira *et al.* (1998) compared the virulence and aggressiveness of 48 strains of *R. solanacearum* isolated from tomato plants in Brazil. Degree of virulence was measured by bacterial wilt index (BWI) and it did not significantly differ among different strains. The aggressiveness measured by IP and LP_{50} showed significant differences among *R. solanacearum* strains.

Based on aggressiveness, different strains of *R. solanacearum* collected from potato field were placed in three groups (McLaughlin and Sequeira, 1989):

Group 1 - weakly aggressive to both susceptible and moderately resistant potato cultivars.

Group 2 - highly aggressive to both potato cultivars.

Group 3 - highly aggressive to susceptible cultivar, but weakly pathogenic to moderately resistant cultivar

Aggressiveness of different strains of *P. solanacearum* obtained from the French West Indies was compared on tomato (Prior *et al.*, 1994). These strains were divided into four profiles of aggressiveness on tomato cultivars Floradel, Capitan and Caraibo, which were susceptible, moderately resistant and resistant, respectively. A link appeared to exist between aggressiveness in Capitan and biovar type. The aggressiveness of *R. solanacearum* race 1 was assessed using colonization index at midstem site (wilt + latent infections) in each strain-tomato cultivar combination, which were earlier described by Prior *et al.* (1994). Based on this, strains were ranked into five aggressiveness patterns differing in behaviour with regard to susceptibility of tomato genotypes, and environmental conditions during experimentation (Darasse *et al.*, 1997).

2.3.1 Mechanism of virulence and aggressiveness

Several studies have shown that exopolysaccharide (EPS) is consistently required for virulence in *R. solanacearum* (Cook and Sequeira, 1991; Denny and Baek, 1991; Kao *et al.*, 1992; Liu *et al.*, 2000). They have shown that EPS mutants were less virulent.

Kao and Sequeira (1991) detected the presence of a gene cluster named *'ops'* required for the coordinated biosynthesis of lipopolysaccharide (LPS) and EPS affecting the virulence of *P. solanacearum*. Multiple virulence genes viz., *egl* coding endoglucanase, *pgl* A coding endopolygalacturonase and *eps* coding exopolysaccharide were identified in tomato involved in the wilt disease (Denny *et al.*, 1990).

Arlat and Boucher (1989) identified a *dsp* DNA region controlling aggressiveness of *P. solanacearum* towards tomato. Tn5 induced mutations carried by three non-pathogenic mutants of *P. solanacearum* were found to be located in this region.

A new *P. solanacearum* gene, *Vsr*B, reported by Huang *et al.* (1993) when inactivated caused a major reduction in the virulence and production of EPS. Later in 1994, Schell *et al.* identified *Vsr*A gene which is required for the normal production of EPS, EXPs (extra cellular proteins) and wilt disease. Allen *et al.* (1997) and Qi *et al.* (1997) characterised *peh* SR gene and *peh* B gene respectively which functioned in the virulence of the pathogen. A novel autoregulator named 3-hydroxypalmitic acid methyl ester (3-OH PAME) was found controlling the expression of virulence genes in *R. solanacearum* (Flavier *et al.*, 1997). *Amp* D has been recently identified in bacterial wilt pathogen which confers ampicillin resistance and virulence (Liu *et al.*, 2000).

2.4 Characterisation of the pathogen

The different isolates/strains of *R. solanacearum* can be grouped based on cultural, biochemical and molecular characterisation.

2.4.1 Cultural and morphological characterisation

The morphological features like shape, size and motility are being used to characterise bacteria. Colony morphology varies with different species, strains and races. The size, colour, form, elevation etc. may be used as the criteria for characterising the different bacterial strains.

The first report on shape and size of *P. solanacearum* was made by Smith (1896) as non-spore forming, non-capsulate, Gram-negative, small rods with polar flagella. All strains of *R. solanacearum* so far reported found to be Gram-negative, motile and rod shaped (He *et al.*, 1983; Swanepoel and Young, 1988; Paul, 1998). However, certain wild types of *R. solanacearum* were found non-motile (Bai *et al.*, 2000).

Variability in colony morphology was first described by Okabe (1949), while making a special reference to pathogenicity. He described them as F-wild types which were fluidal, irregularly milky colony, OP – opalescent circular homogenous, C – circular, light brownish, striated and SS – pale, fluorite green

with cream coloured centre. Later, Kelman (1954), observed three main colony types: normal or wild type which were irregularly round, fluidal and opaque. In contrast, the mutant type formed uniformly round, butyrous and translucent colonies. In both types, colony surface was smooth. A third type colony was butyrous translucent colonies with a rough surface. Several others also noticed the similar colony types with bacterial isolates from different sources (Husain and Kelman, 1958; He *et al.*, 1983; Mathew and Nayar, 1983; Prior and Steva, 1990; Paul, 1998).

Khan *et al.* (1979) made a comparative study on the morphological and cultural characters of the isolates of *P. solanacearum* obtained from brinjal, chilli, tomato and potato on TZC medium. The colonies of the isolates from chilli and potato were found fluidal, slightly convex with slight pinkish centre whereas that from brinjal and tomato were highly fluidal, convex to flat with pinkish centre. The absence of pigment production by *P. solanacearum* on King's B medium had been reported by many workers (Smith, 1914; Hayward, 1964; Nayar, 1982; Swanepoel and Young, 1988; Jyothi, 1992). Paul (1998) isolated the pathogen from brinjal, chilli and tomato on TZC medium and found that all the isolates produced circular, smooth, raised, creamish white colonies with pink centre and entire margin. The fluidity was highest with brinjal isolate followed by tomato and lowest in chilli. However, Mathew *et al.* (2000) reported that the slime production and fluidity were more in brinjal and chilli isolates compared to tomato isolate.

2.4.2 Biochemical characterisation

Biochemical tests are carried out to detect the presence of certain enzymes like cytochrome-c-oxidase, catalase, arginine dihydrolase and lipase, pigment production; levan production; gas production from nitrate, glucose oxidation etc. These tests help in the characterisation and grouping of the different isolates.

Determination of the Gram reaction by staining is the essential first step in the characterisation and classification of bacteria from any source. Suslow

et al. (1982) developed a simple test, i.e., solubility in 3 per cent KOH solution and its result correlated very well with the staining reaction.

2.4.2.1 Enzyme activity in the bacteria

Based on the presence or absence of cytochrome oxidase, Kovacs (1956) divided aerobic or facultatively anaerobic bacteria into two groups, oxidase positive and negative. He attempted to differentiate among pseudomonads and classified *P. solanacearum* as oxidase positive. Catalase activity was also detected with *P. solanacearum.* Sierra (1957) developed a simple method for the detection of lipolytic activity of microorganisms. Hydrolysis of Tween 80 was considered as the indication of lipolytic activity. Based on the arginine dihydrolase reaction, Thornley (1960) differentiated *Pseudomonas* from other Gram-negative bacteria. He clearly distinguished those species which were arginine dihydrolase positive (P. *aeruginosa* and *P. fluorescens)* and those unable to produce ammonia from arginine under anaerobic conditions (P. *syringae* and *P. solanacearum).* Based on the biochemical tests carried out by many workers, different isolates of *R. solanacearum* were identified as oxidase, catalase and lipase positive; arginine dihydrolase negative (He *et al.*, 1983; Swanepoel and Young, 1988; Prior and Steva, 1990; Jyothi, 1992; Paul, 1998; Mathew *et al.*, 2000).

2.4.2.2 Pigment production

King *et al.* (1954) developed medium B for the production of fluorescent pigment. They concluded that *P. solanacearum* produced no fluorescent pigment, but brown diffusible melanin like pigments on the medium B containing 1 per cent tyrosine. Hayward (1964) confirmed the production of a diffusible brown pigment on tyrosine medium, the intensity of which varied between the isolates.

Production of melanin pigments by *P. solanacearum* strains was tested on casamino acids – peptone glucose (CPG) medium containing 0.1per cent tyrosine. All strains, except a few, produced brown melanin pigment (He *et al.*,

1983; Prior and Steva, 1990). Absence of fluorescent pigment on King's B medium has been reported by several workers (Ile *et al.*, 1983; Prior and Steva, 1990, Swanepoel and Young, 1988).

2.4.2.3 Levan production

Production of levan from sucrose is one of the biochemical tests for characterisation of *R. solanacearum.* Hayward (1964) reported the production of levan (poly-fructose) by some fluorescent pseudomonads and *P.solanacearum* did not produce levan. However, He *et al.* (1983) found that most of the strains of *P. solanacearum* from China produced levan. Similarly, the isolates obtained from brinjal, chilli and tomato produced levan in the peptone beef extract medium containing five percent sucrose (Paul, 1998). According to Prior and Steva (1990), some variations in the levan production occurred among strains of *P. solanacearum* from French West Indies.

2.4.2.4 Nitrate metabolism

Nitrate metabolism varied with biovars (Hayward *et al.*, 1990). The results of gas production from nitrate (denitrification) were most reproducible when a semisolid succinate/nitrate or glycerol/nitrate medium was used. Most of the strains of *R. solanacearum* were found reducing nitrate, either with or without the production of gas (He *et al.*, 1983; Prior and Steva, 1990; Swanepoel and Young, 1988; Mathew *et al.*, 2000).

2.4.2.5 Glucose metabolism

A simple test to differentiate between oxidative and fermentative metabolism of glucose was developed by Hugh and Leifson (1953) and it was found that *P. solanacearum* was oxidative. He *et al.* (1983) tested oxidative and fermentative metabolism of *P. solanacearum* strains from China in Hayward's medium containing glucose in plugged test tubes both sealed and unsealed and found that in unsealed tubes, the reaction was positive and negative in the sealed tubes indicating aerobic nature. South African strains of *P.solanacearum* were

found oxidative when tested for oxidation/fermentation of glucose (Swanepoel and Young, 1988). Based on this test, Paul (1998) proved the aerobic nature of *R. solanacearum* isolated from brinjal, chilli and tomato.

2.4.3 Biovar identification

A biovar/biotype is a subgroup within a species usually characterized by the possession of a single or a few characters in common. The different isolates of *R. solanacearum* can be grouped into biovars based on the utilisation of disaccharides and hexose alcohols.

Hayward (1964) classified the isolates of *P. solanacearum* from different host plants and different parts of the world into four biotypes based on the capacity to utilize disaccharides viz., lactose, maltose and cellobiose and hexose alchohols viz., mannitol, sorbitol and dulcitol. Biotype 1 oxidised neither group, biotype 2 only disaccharides, biotype 3 both the groups and biotype 4 only hexose alcohols. Later He *et al.* (1983) identified certain isolates from China which oxidised mannitol, but not sorbitol or dulcitol and designated them as biotype 5.

Most of *R. solanacearum* strains affecting solanaceous crops belong to biovar III (Hayward, 1964; He *et al.*, 1983; Prior and Steva, 1990; Paul, 1998; Mathew *et al.*, 2000).

In Kerala, Devi (1978) classified all the isolates obtained from tomato, brinjal and chillies as biovar IV. Jyothi (1992) characterised *P. solanacearum* from ginger also, apart from brinjal, chilli and tomato and reported that they belonged to biovar III.

The 12 isolates of *P. solanacearum* affecting solanaceous vegetables were collected from Himachal Pradesh and among these, chilli isolate was identified as biovar V, five of the six tomato isolates, one of the three brinjal isolates and potato isolate belonged to biovar III group. Other four isolates of brinjal and tomato differed from others in its inability to utilize/oxidize dulcitol and was

considered as a sub-type in biovar III and designated as biovar III A (Kumar *et al.*, 1993). Paul (1998) identified certain tomato and chilli isolates as biovar III and those from brinjal as biovar V. Mathew *et al.* (2000) reported biovar 3 affecting brinjal, chilli and tomato and biovar 5 in tomato.

2.4.4 *Hypersensitivity reaction (HR)*

Hypersensitivity is an extreme degree of susceptibility in which rapid death of the cells in the vicinity of the invading pathogen occurs. This halts the progress of the pathogen although it may not die immediately. Hypersensitivity, thus, is a sign of very high resistance approaching immunity. Based on HR, the isolates can be grouped into races. Race is a genetically and often geographically distinct mating group within a species. It is also considered as a group of pathogens that infect a given set of plant varieties. Based on the reaction of pathogenic bacteria on plants like tobacco/capsicum, they are grouped into races.

Several attempts had been made to group *P. solanacearum* isolates into races based on the difference in physiological and morphological characteristics (Kelman, 1954; Buddenhagen and Kelman, 1964; Hayward, 1964) and pathogenicity (Kelman and Person,1961).

Based on host ranges, Buddenhagen *et al.* (1962) grouped different isolates into three races. Race 1 affected tobacco, tomato, many solanaceous and other weeds and certain diploid bananas. Race 2 caused bacterial wilt of triploid bananas, *Heliconia* or both. Race 3 affected potatoes and tomatoes, but is not highly virulent on other solanaceous crops.

Hypersensitive reaction between the plant pathogenic bacteria and resistant host plants was discovered to be the most universal defense mechanism in plants (Klement *et al.*, 1964). Based on HR, Lozano and Sequeira (1970) differentiated three races of *P. solanacearum* by a leaf infiltration technique in tobacco. Race 1 caused no visible symptoms on inoculated leaves after 24 h, but a dark brown necrotic lesion surrounded by a yellow halo appeared after 36 h in the infiltrated area. By 60 h, the bacteria had invaded the adjoining tissues and

vascular elements. After eight days, extensive wilting, yellowing and necrosis of the leaf tissues resulted. Race 2 produced water soaked area after 10 to 12 h followed by light yellowing. By 60th h, the affected area become papery and dried up. This was the typical HR on tobacco. Race 3 isolates caused only a yellowish discoloration of the infiltrated area by 48 h after inoculation.

Persley *et al.* (1985) grouped the bacterial wilt pathogen into five races which differed in host ranges, geographical distribution and ability to survive under different environmental conditions.

1. Race 1 (Solanaceous strain): wide host range, distributed throughout the lowlands of tropics and subtropics.

2. Race 2 (Musaceous strain): restricted to Musa and a few perennial hosts initially limited to American tropics, now spreading to Asia.

3. Race 3 (Potato strain): restricted to potato and a few alternative hosts in the tropics and subtropics.

4. Race 4 (Ginger strain): from Philippines

5. Race 5 (Mulberry strain): from China

He *et al.* (1983) reported that most strains of *P. solanacearum* from China belonged to race 1. The 23 out of 24 strains of *P. solanacearum* collected from French West Indies were placed in race 1; the exception was placed in race 3 (Prior and Steva, 1990). Akiew and Hyde (1993) reported the first detection of *P. solanacearum* race 2, strain SFR on *Heliconia* in Australia. Based on melanin like pigment formation in a tyrosine containing medium and HR on tobacco leaves, Rahman *et al.* (1996) classified 14 out of 17 isolates of *R. solanacearum* from Bangladesh affecting chillies, eggplant, potato and tomato plants as race 1 and rest three as race 3. Samaddar *et al.* (1998) identified *R. solanacearum* affecting aubergine, tomato, potato and chilli collected from West Bengal as race 1. Horita and Tsuchiya (2000) classified Japanese and foreign strains of

R. solanacearum into races 1 and 3 based on 16S ribosomal RNA gene sequences. The isolates of *R.solanacearum* affecting solanaceous vegetables in Kerala were classified as race 1 based on hypersensitive response on bell pepper (Mathew *et al.*, 2000).

A genetic approach has also been undertaken to study the race differentiation based on the HR on tobacco.

Boucher *et al.* (1987) clustered the *P. solanacearum* genes controlling pathogenicity on host plants and hypersensitivity response on non host plants and designated as *hrp* genes. He observed that a second group of genes interfered with disease development on host plants, but which were not involved in the induction of HR on non-host plant species and were referred to as *dsp* (disease specific). The third group corresponds to avirulence genes *(avr)*. These genes were involved in the induction of the HR on non-host plant species.

During an incompatible reaction between tobacco and *R.solanacearum*, two classes of genes viz., *hsr* (hypersensitivity related) genes activated preferentially during the HR reaction and *str* (sensitivity related) genes expressed strongly during both compatible and incompatible interactions have been identified (Czernic *et al.*, 1996). Arlat *et al.* (1994) identified a new class of extracellular bacterial proteins, *Pop* A1, which acted as specific HR elecitors. Thus, certain genes and proteins were identified which involved in HR reaction.

2.4.5 Molecular characterisation

Advances in the field of molecular genetics have led to the establishment of different types of molecular markers like isozyme and DNA. Protein and DNA markers together constitute molecular markers. Several DNA markers have been developed in recent years based on the extensive variation at DNA level among the natural populations. These markers could be applied successfully in the detection and identification and plant pathogenic bacteria. Most important among these are RFLP (Restriction Fragment Length Polymorphism) and PCR

(Polymerase Chain Reaction) based RAPD (Randomly Amplified Polymorphic DNA) (Singh, 1998).

2.4.5.1 RFLP

RFLP is based on the specificity of enzymes called restriction endonucleases and probes used to detect alterations in different strains/species in the positions of recognition sites of the given enzyme in the chromosome region identified by the probe. The enzymes bind at the recognition sites and cut the DNA; thus producing fragments of different lengths from the same stretch of genomic DNA of different strains or related species. These differences are detected as bands differing in mobilities following Southern hydridization.

A few studies have been carried out in bacteria using RFLP as the molecular marker. Boucher *et al.* (1987) developed a method of isolation of genomic DNA from bacterial cells and constructed *Eco*RI restriction maps of *P. solanacearum* and *Xanthomonas campestris* strains. They confirmed that, *hrp* genes present on the plasmid (pVir2) deciding the pathogenicity and hypersensitivity on tobacco were clustered. Southern hybridization could demonstrate the existence of both structural homology of *hrp* cluster of pVir2 with all the pathovars of *X. campestris* tested and the common pathogenicity genes among *P. solanacearum* and *X. campestris* pathovars.

Southern hybridization of various strains of *P. solanacearum* was carried out after DNA restriction with *Eco*R1(Seal *et al.*, 1992). This was the first report describing a DNA probe specific for the species of *P.solanacearum*. Only a few strains hybridized with the DNA probe. Genetic diversity within *P. solanacearum* was determined by genomic digestion with rare cutting endonuclease enzyme (Smith *et al.*, 1994). The resultant DNA fragments were separated by pulsed field gel electrophoresis. The method was suitable for analysis of races 1, 2 and 3 and for ecological studies on closely related bacteria.

Taghvi *et al.* (1994) made some phenotypic and molecular approaches to strain differentiation in *P. solanacearum* associated with groundnut bacterial wilt.

Using RFLP analysis, grouping of isolates was done which correlated with their phenotypes.

RFLP analysis was also done to distinguish between the strains of *R.solanacearum* causing Moko disease of *Musa spp.* (Gough *et al.*, 1995).

PCR-RFLP analysis of *rrn* operons (ribosomal operons) has been found as a reliable technique to evaluate the genetic relatedness on a large number of strains. The data obtained by RFLP analyses of the *rrn* genes showed that *Pseudomonas syringae* and *Pseudomonas viridiflava* belonged to the same genomic group (Manceau and Horvais, 1997).

Twelve strains of *R. solanacearum* representing all biovars and races were subjected to digestion by 37 different restriction enzymes. RFLP could characterise the strains of race 2 and biovars 3 and 4. However, the strains of race 1 and race 3 could not be differentiated (Roncal *et al.*, 1999).

Poussier *et al.* (2000) assessed the genetic diversity among the strains of *R. solanacearum* by PCR-RFLP method. The PCR-RFLP clustering agreed with the biovar classification and the geographical origin of the strains. RFLP analysis of PCR-amplified 16S rDNA could efficiently differentiate both Japanese strains and three types of foreign strains which differed in biovar and geographic origin (Horita and Tsuchiya, 2000).

The plasmid DNA of different strains of *R. solanacearum* were also subjected to RFLP analysis to characterise the strains. Morales and Sequeira (1985) digested the plasmid DNA from three different strains with *Bam* H1 and *Eco*R1 and obtained very different fragment patterns. They found no apparent relationship between the presence of plasmids and host of origin, natural resistance to antibiotics or geographic origin of the strains.

The different strains were grouped based on digestion of their plasmid DNA. Elumalia and Mahadevan (1997) digested the plasmids of ten strains of *R.*

solanacearum with *Eco*R1 and revealed that plasmids of race 3 strains clustered in one group and those of race 1 strains clustered in a separate group.

2.4.5.2 PCR based RAPD

a. PCR

Kary Mullis discovered Polymerase Chain Reaction in 1985(Saiki *et al*, 1988), which has revolutionised molecular biology studies. It is the amplification of a DNA fragment into millions of copies using a short DNA oligomer as a primer. The two oligonucleotide primers flank the DNA fragment by hybridizing to the opposite strands of the target DNA.

The DNA replication involves polymerisation of nucleotides using a template DNA strand with the help of an enzyme, DNA polymerase. Amplified DNA is obtained by repeating the reaction, i.e., by regular denaturation of freshly synthesized double stranded (ds) DNA molecules by heating at 90 to 98°C. Then annealing of primers takes place at about 40 to 60°C followed by the extension of newly formed DNA strand at 70 to 75°C. This process is automatically repeated 20 to 30 times so that a billion copies of the sequence flanked by the left and right primers can be produced.

Using PCR, DNA polymorphism can be studied at loci with known DNA sequence using the primers of known sequence. Genetic diversity among different strains of bacteria can be assessed using PCR and the polymorphism obtained using certain specific primers can be correlated with the geographical origin.

Smith *et al.* (1995) determined the genetic diversity among the isolates of *R. solanacearum* race 3 biovar II of Kenya by PCR with repetitive sequences. Among 46 isolates collected from the major potato growing regions of Kenya, 45 were identified as race 3 biovar II and one belonged to race 3 biovar N2 using PCR technique. PCR was used to characterise *R. solanacearum* isolates from

different plants including hibiscus, tomato, sugarcane, capsicum and coffee by PCR amplification (Fernandez *et al.*, 1996).

A novel method for species and strain specific DNA probes and PCR primers for identifying *R. solanacearum* was developed by Opina *et al.* (1997). Its feasibility and reliability for detection and identification of the bacteria was tested in several countries. PCR had been used as reliable and sensitive method to detect even low-level infection in potato tubers by *R.solanacearum* (Seigner, 1997).

Semiselective medium and PCR technique were combined to detect the viable cells of *R. solanacearum* in soil (Ito *et al.*, 1998). DNA extracted from 92 strains of soil bacteria including *R. solanacearum* were subjected to PCR. A 281 bp fragment was amplified only from DNA of *R. solanacearum* strains. PCR amplification of other strains generated products in varying pattern, like single band with different sizes, several DNA bands and even without any bands.

Repetitive element sequence based PCR (rep PCR) has also been used to determine the genetic diversity among the 43 isolates of *R. solanacearum* race 3 biovar II of European origin (Smith *et al.*, 1998). Several other workers had carried out PCR to determine the variability among the strains of *R. solanacearum* (Hartung *et al.*, 1998; Seal *et al.*, 1999; Boudazin *et al.*, 1999). PCR amplified fragments using primers P759/P760 were subjected to restriction analysis corresponding to biovar/race combinations of *R. solanacearum*. This method could perform the rapid identification of *R. solanacearum* (Roncal *et al.*, 1999).

Specific primers for the detection of *R. solanacearum* in soil samples were designed by Lee and Wang (2000). These primers amplified the same 1.1 kb PCR product from all *R. solanacearum* strains and failed to amplify DNA from any other bacterial strain. A fluorogenic (TaqMan) PCR assay was developed to detect *R. solanacearum* strains where two fluorogenic probes were utilized in a multiplex reaction. The specificity and sensitivity of the assay

combined with high speed and reliability offer potential advantages in detecting the presence of *R. solanacearum* from any plant material (Weller *et al.*, 2000).

b. RAPD

RAPD is a modification of basic PCR technique. Instead of using a pair of carefully designed and fairly long oligonucleotide primers to amplify a specific target sequence, a single short oligonucleotide primer, which binds to many different loci is used to amplify random sequences from a complex DNA template. The number of amplified products depends on the length of the primer and size of the target genome and is based on the probability that a given DNA sequence (complementary to that of the primer) will occur in the genome on opposite strands of the DNA, in opposite orientation within a distance readily amplifiable by PCR. Primers are generally of random sequence, which contain atleast 50 per cent Gs and Cs and lack internal inverted repeats. Products are easily separated by electrophoresis method visualised by UV with ethidium bromide stained gels.

Similar to PCR, the polymorphism obtained by RAPD can be used to determine the genetic diversity among different bacterial strains. Opina *et al.* (1997) differentiated three genetically diverse strains belonging to biovars 2, 3 and 4 using RAPD technique. RAPD analysis was used to examine genomic differences between 18 isolates of *R. solanacearum* collected from nine different fields in Japan and could detect the genomic diversity among the isolates (Ito *et al.*, 1998). Several plant pathogenic bacteria like *Xanthomonas* spp., *Clavibacter* spp., *Erwinia amyovora* and *R. solanacearum* could be identified by RAPD assay. These bacteria were screened using 30 synthetic decamer oligonucleotide primers which allowed the differentiation of species, subspecies and pathovars (Salava *et al.*, 1998).

Thwaites *et al.* (1999) used RAPD and rep PCR based finger printing to investigate the genetic relationship between strains of *R. solanacearum* causing

vascular wilt diseases of *Musa* spp. The isolates of *R.solanacearum* could be differentiated into three groups based on polymorphism obtained.

Recently, Lee and Wang (2000) used RAPD method to design the specific primers for detecting *R. solanacearum* in soil samples by PCR. The RAPD procedure was used to find a specific 0.7 kb DNA fragment unique to *R. solanacearum* which was then used as a probe to identify a 2.7 kb *Eco*R1 fragment from another strain.

2.4.5.3 Plasmid profile

Plasmids are autonomously replicating genetic elements existing in multicopies in the bacterial cell in a stable form under non-selective conditions. In plant pathogenic bacteria, the important traits like virulence, production of toxins, enzymes, resistance to antibiotics and host range are coded by plasmids. *Hrp* genes which are normally chromosomal, were found located on a megaplasmid in *P. solanacearum* (Rosenberg *et al.*, 1982).

The different strains of *R. solanacearum* may harbour 1 to 3 plasmids, but most of the strains contain a single plasmid. Morales and Sequeira (1985) reported that 22 out of 39 strains of *P. solanacearum* harboured 1 or 2 plasmids with relative masses ranging from 5 to more than 500 mega daltons. Elumalai and Mahadevan (1997) isolated *R. solanacearum* strains from different parts of India and could find that they harboured 1 to 3 plasmids.

A rapid alkaline extraction procedure for screening recombinant plasmid DNA was developed by Birnboim and Doly (1979). It is based on the principle that between pH 12 and 12.5, linear DNA gets denatured, whereas covalently closed circular (CCC) DNA remains intact. Kado and Liu (1981) extracted plasmid DNA by alkaline lysis of the cells followed by phenol chloroform extraction. In another extraction procedure, the cells were lysed directly in wells formed in agarose gels before electrophoresis (Comai and Kosuge, 1982). Morales and Sequeira (1985) slightly modified the method employed by Kado by replacing Sodium Dodecyl Sulfate (SDS) with 3 per cent sarcosyl in lysing

solution. The isolation procedure of mega plasmid from *P.solanacearum* having slight modification over the above had been reported by Boominathan and Mahadevan (1988).

2.5 Role of plasmids in EPS production and virulence

Exopolysaccharide (EPS) is an important factor required for virulence and this trait is often encoded by plasmids.

Wilting incited by *P. solanacearum* was found largely due to the occlusion of xylem vessels by EPS (Cook and Sequeira, 1991). Genetic evidence that EPS is consistently required for the wild type of virulence in *R.solanacearum* has been provided by Denny and Baek, 1991; Kao *et al.*, 1992; Araud *et al.*, 1998 and Liu *et al.*, 2000. However, certain highly virulent strains of *Ralstonia solanacearum* were found defective in EPS production (Xu *et al.*, 1990).

The *eps* gene cluster of *R. solanacearum* was characterised by Huang and Sehell (1995). Certain *eps* genes are found involved in the production of EPS. Products of *Eps* R synthesize EPS and its expression is under the control of several proteins (McWilliams *et al.*, 1995 and Chapman and Kao, 1998).

Boucher *et al.* (1986) and Arlat *et al.* (1990) confirmed the presence of virulence genes on megaplasmid of *P. solanacearum* GMI 1000. The culture age was found to influence greatly on the plasmid of *P. solanacearum* (Rani *et al.*, 1989). If grown on catechin, plasmid DNA was maximum in the stationary phase of 36 h old culture and least in the cells from the lytic phase of 48 h old.

Several studies have been carried out on genetic basis for conversion of virulent to nonvirulant colonies (Morales and Sequeira, 1985; Denny *et al.*, 1994). However, conversion from nonmucoid to mucoid colonies has also been reported (Shekhawat *et al.*, 1992). Since the occurrence of a single plasmid with different mobilities was observed both in mucoid and non mucoid forms, role of plasmid in this type of phenotypic conversion remains unknown.

Plasmid DNA of the certain strains of *P. solanacearum* could also be utilised in the construction of stable cloning vectors for this species (Morales and Sequeira, 1985).

Thus, the molecular characterisation has been found to depict more variation among the bacterial isolates at genetic level compared to cultural and biochemical characterization. Hence the present study has been carried out to characterise the isolates of *R. solanacearum* at molecular level.

Materials and Methods

3. MATERIALS AND METHODS

The present study was conducted at the Department of Plant Pathology and Centre for Plant Biotechnology and Molecular Biology, in the College of Horticulture, Vellanikkara during the period, 1999-2001. A pot culture experiment was taken up during September to December 2000 and other experiments were conducted in the lab.

3.1. Collection of the bacterial wilt affected plants

Bacterial wilt affected brinjal, chilli and tomato plants were collected from three different locations viz., Vellanikkara, Kumarakom and Ambalavayal, representing three agro climatic zones of Kerala (Central zone, Special problem area zone and High range zone). These were subjected to ooze test to confirm presence of bacteria. An oblique sharp cut was made at basal part of the stem and the cut end was placed in a test tube containing 5 ml of sterile water and later observed for streaming out of ooze from xylem vessels.

3.2. Isolation and maintenance of *Ralstonia solanacearum*

Basal stem portion of the wilted plant samples was first surface sterilized with 70 per cent ethyl alcohol, made into small pieces of 10-15 mm using a sterilized razor blade, and kept suspended in 5 ml of sterile distilled water in a test tube for 10 minutes to get a turbid suspension. The bacteria was then isolated on triphenyl tetrazolium chloride (TZC) medium (Kelman, 1954) by streaking with a loopful of inoculum and incubated at room temperature for 48 h. Slimy fluidal cream coloured colonies with light pink centre were selected and then purified by streaking again on the same medium. Composition of TZC medium is given in Appendix I.

Koch's postulates were proved on respective host plants using the method standardised for inoculation technique.

Three to five characteristic single colonies were selected and maintained at room temperature by suspending in 5 ml sterile water in screw-capped test tubes for subsequent use. (He *et al.*, 1983).

A total of nine isolates three from each selected location were used for the study. The details of these isolates and locations are given below.

Table3.1 Details of isolates of *R. solanacearum* collected from different locations

Sl. No.	Isolate	*Locality from which it was collected*	Host plant
1	V1	College of Horticulture, Vellanikkara	Brinjal
2	V2	College of Horticulture, Vellanikkara	Chilli
3	V3	College of Horticulture, Vellanikkara	Tomato
4	K1	Regional Agrl. Research Station, Kumarakom	Brinjal
5	K2	Regional Agrl. Research Station, Kumarakom	Chilli
6	K3	Regional Agrl. Research Station, Kumarakom	Tomato
7	A1	Regional Agrl. Research Station, Ambalavayal	Brinjal
8	A2	Regional Agrl. Research Station, Ambalavayal	Chilli
9	A3	Regional Agrl. Research Station, Ambalavayal	Tomato

All isolates were tested periodically for purity and virulence by streaking on TZC medium.

3.3 Standardisation of inoculation technique

A pot culture study was carried out to standardize inoculation technique in brinjal, chilli and tomato using the following highly susceptible varieties.

Brinjal : Pusa Purple Long

Chilli : Pusa Jwala

Tomato : Pusa Ruby

The seedlings were raised in sterilized soil, then transplanted to small plastic pots of size 5 cm^3, each containing 50 g sterile soil. Thirty days old, vigorously growing seedlings with 5 to 6 leaves were used for the inoculation studies.

Since preliminary experiment with bacterial culture resulted in low wilt incidence, the fresh bacterial ooze from wilted plants of brinjal, chilli and tomato was used for inoculation. The bacterial ooze was collected from wilted plants of Vellanikkara in 100 ml of sterile distilled water and the optical density (OD) of the suspension was adjusted to 0.3 at 600 nm containing 10^8 cfu/ml. The ooze from respective host plants was used as standard inoculum for all experiments. Five different methods were tried for inoculation viz., leaf clipping, stem puncturing, root dipping, soil drenching and soil drenching with wounding.

Experiment was replicated four times with 12 plants for each isolate along with a control.

3.3.1 Leaf clipping

Three to four cuts were made across the veins on leaf blade of the top four fully opened leaves of the healthy plants with a pair of sterilized scissors dipped in the bacterial suspension.

3.3.2 Stem puncturing

A drop of bacterial suspension was placed in the axils of second and third expanded leaves below the stem apex and then a pin was forced into the stem through

the drop. A piece of cotton dipped in the bacterial suspension was then placed in the punctured axils.

3.3.3 Root dipping

Seedlings were lifted carefully and roots washed with sterile distilled water to remove the soil particles. Then roots were dipped in the bacterial suspension prepared as in 3.3, for 30 minutes and seedlings transplanted in pots containing sterilized soil.

3.3.4 Soil drenching

The soil in the pot was drenched by pouring 30 ml of inoculum around the base of the seedling.

3.3.5 Soil drenching with wounding

The lateral roots of the seedlings were cut along one side of the plants by inserting a knife, 1.5 cm away from the collar region, to a depth of about 4 cm. Soil was then drenched with 30 ml of the inoculum around the base of the seedling.

Inoculated plants were observed daily upto 12 days, for development of symptoms. Incubation period (IP), per cent wilt incidence and bacterial wilt index (BWI) were calculated for each treatment as follows.

IP = number of days before plants have developed visible symptoms.

$$\text{Per cent wilt incidence} = \frac{\text{number of wilted plants}}{\text{Total no. of plants inoculated}} \times 100$$

Calculation of BWI is given under 3.4.1

The best inoculation technique was selected based on the above three parameters.

3.4 Comparison of virulence and aggressiveness.

Virulence and aggressiveness of all nine isolates were tested on respective hosts with highly susceptible varieties mentioned under 3.3. Pot culture experiment was taken up to assess the virulence and aggressiveness of the isolates.

The design adopted was CRD with four replications, three plants in each replication, for the nine treatments.

Inoculation was done using the method standardised for the host, as described in 3.3, at 5 to 6 leaf stage of the respective host plants. The bacteria was then reisolated from the artificially inoculated plants and compared with the original isolate to ensure the identity of the pathogen (Winstead and Kelman, 1952).

3.4.1 Virulence

For assessing the virulence of isolates, plants inoculated with *R.solanacearum* were scored on 0 to 5 scale adopted from Winstead and Kelman (1952) which is given below.

0 - no symptoms

1 - one leaf partially wilted

2 - two to three leaves wilted

3 - all except upper two to three leaves wilted

4 - all leaves wilted

5 - plant dead

Scoring was done daily for 15 days and virulence was expressed as bacterial wilt index (BWI) according to Emping *et al.* (1962).

$$BWI = \frac{\text{Sum of all readings per treatment}}{\text{Total no. of plants per treatment x maximum disease score}} \times 100$$

Disease index rating was done as follows as suggested by Kelman and Person (1961) with slight modifications:

H - Highly virulent (>65%)

M - Moderately virulent (40-65%)

W - Weakly virulent (< 40%)

A - Avirulent (0)

3.4.2 Aggressiveness

Aggressiveness was measured by incubation period (IP) and latent period (LP_{50}) according to Adhikari (1993). Plants were observed daily for 15 days.

IP = No. of days for developing visible symptoms

LP_{50} = No. of days for 50% wilting of plants

Based on these, the virulence and aggressiveness of the nine isolates were compared.

3.4.3 Statistical analysis

The statistical analysis was done using Mstat C package.

3.5 Cross inoculation of the isolates

A pot culture experiment was carried out for the cross inoculation studies of different isolates. Studies were conducted by cross inoculating brinjal, chilli and tomato with the standard bacterial inoculum collected from wilted plant.

Treatments were as follows:

1. Brinjal isolate on chilli
2. Brinjal isolate on tomato
3. *Chilli isolate on brinjal*
4. Chilli isolate on tomato
5. Tomato isolate on brinjal
6. Tomato isolate on chilli

There were four replications for each treatment. Plants were observed for development of wilt symptoms and observations were taken on disease score and incubation period. BWI was calculated as mentioned in 3.4.1. Wilted plants were subjected to ooze test for the confirmation of infection.

3.6 Characterisation of *Ralstonia solanacearum*

Isolates of *R. solanacearum* isolates were characterised by cultural, morphological, biochemical and molecular characters.

Composition of media used for various tests are given in Appendix I.

3.6.1 Cultural and morphological characterisation

The suspension of each isolate was prepared in 5 ml of sterile distilled water. A loopful of this inoculum was streak plated on Kelman's TZCmedium to get single discrete colonies. The plates were then incubated at room temperature for 48 h.

Isolated colonies were observed for the following characters.

1. Size : Pinpoint, small, moderate or large.
2. Pigmentation : Colour of colony

3. Form : The shape of colony.

   a. Circular : Unbroken peripheral edge

   b. Irregular : Indented peripheral edge

   c. Rhizoid : Root like spreading growth

4. Margin : The appearance of the outer edge of the colony.

   a. Entire : Sharply defined, even

   b. Lobate : Marked indentations

   c. Undulate : Wavy indentations

   d. Serrate : Tooth like appearance

   e. Filamentous : Thread like, spreading edge

5. Elevation : The degree to which colony growth is raised on the agar surface.

   a. Flat : Elevation not discernible

   b. Raised : Slightly elevated

   c. Convex : Dome-shaped elevation

   d. Umbonate : Raised with elevated convex central region

6. Mucoid nature : Amount of slime produced.

   a. Mucoid

   b. Non-mucoid.

3.6.2 Staining

Gram staining was done for the morphological characterisation. It revealed the shape of the cell.

3.6.3 Motility

Motility of the isolates was tested using cavity slide. A small drop of bacterial suspension was placed on a clean cover slip. The cover slip was then carefully inverted and placed over the cavity slide such that the drop of bacterial suspension was hanging into the cavity of the slide. It was observed under the microscope to observe motility of bacterial isolates.

3.6.4 *Biochemical characterisation*

The following tests were conducted with all the nine isolates for biochemical characterisation. 48 h old bacterial cultures were used for different tests unless and otherwise specified.

3.6.4.1 Solubility in 3 per cent KOH

Two drops of 3 per cent KOH were placed at the centre of a glass slide. A loopful of bacterial growth was removed from the agar surface with an inoculation loop and placed in the alkali with rapid circular agitation. After five to eight seconds, the loop was alternately raised and lowered just off the slide surface to detect the presence or absence of viscous strand formation. (Suslow *et al.*, 1982).

3.6.4.2 Kovacs' oxidase test

Ready to use oxidase discs from Hi-Media, Mumbai were used for this test. A disc was placed at the centre of glass slide. A loopful of inoculum was taken and rubbed on the disc. Time taken for development of purple colour, if any, was noted (Kovacs, 1956).

3.6.4.3 Catalase test

Hydrogen peroxide (3 per cent) was added to 24 h bacterial cultures growing on Luria agar slants. Formation of bubbles indicate positive reaction (Cappuccino and Sherman, 1992).

3.6.4.4 Fluorescent pigment

Production of fluorescent pigment by the bacterial isolates was tested by streaking them on King's B agar (King *et al.*, 1954), containing 1per cent tyrosine. Plates were incubated at 28°C for 48 h. Plates were then observed under UV light for the production of fluorescent pigment or brown diffusible melanin pigment.

3.6.4.5 Levan from sucrose

Single colonies of bacterial cultures were streaked on King's B agar plates supplemented with 5 per cent sucrose. After incubation at 28° C for 48 h, they were examined for levan production, as indicated by raised, convex colonies (Hayward, 1964).

3.6.4.6. Arginine dihydrolase reaction

Thornley's semisolid medium (Thornley, 1960) was used for this test. The basal medium was heated at 100° C with periodic swirling to melt the agar and dispensed the molten medium in 5 ml quantities into 15 ml capacity screw capped test tubes and then autoclaved at 121° C at 15 psi. It was allowed to set at room temperature. The medium was inoculated by stabbing with sterile bacterial loop containing inoculum to the base of the tube. After sealing the medium with 3 ml molten 1 per cent agar at 45° C, the tubes were incubated at 28° C for 7 days. Any colour change indicative of change in pH under the agar seal was observed.

3.6.4.7 Oxidation of glucose

Hayward's semisolid basal medium was used for this test. The constituents were dissolved and pH adjusted to 7.0 with 40 per cent NaOH so that colour of the medium was olivaceous green. Bacto agar at 0.3 per cent was added to basal medium. The medium was autoclaved at 121° C at 15 psi.

Glucose solution was prepared separately by adding 1 g of glucose to 10 ml of sterile distilled water and then heating the solution at 100° C for 30 minutes to ensure sterility. This solution was added to 100 ml of the basal medium after melting and cooling to about 60 to 70° C and then mixed by rotation. The medium was dispensed at 5 ml per tube in sterile screw capped test tubes and allowed to solidify.

A loopful of inoculum was picked up from TZC medium using a sterilized bacterial loop and stab inoculated to the base of the medium in a tube. Each isolate was inoculated in 2 tubes. One tube was then sealed with 3 ml of molten sterile 1 percent agar cooled to 45° C. Duplicates of both sealed and unsealed were maintained. All tubes were incubated at room temperature and examined after 24, 48 h and 7 days for any colour change of the medium (Hugh and Leifson, 1953).

3.6.4.8 Denitrification test

Bacterial isolates were tested for gas production from nitrate using two media viz., Van den Mooter Succinate (VMS) and Van den Mooter Glycerol (VMG) described by Van den Mooter (1987). The former medium contains succinate as the major carbon source and the latter contains glycerol.

The media constituents were dissolved in 100 ml water, 0.3 g agar was added and autoclaved. Five ml medium was dispensed into sterile screw capped test tubes. The medium was inoculated by stabbing each isolate in duplicate and sealed with molten sterile 1 per cent agar at 45° C. The tubes were incubated at room temperature

for 7 days with daily observation for the production of gas under the seal (Hayward *et al.*, 1990).

3.6.4.9 Lipase activity

The medium described by Sierra (1957) was used for the lipase test. A 10 per cent stock of Tween 80 was sterilized separately and added at 10 ml per 100 ml of molten basal medium. It was then poured into sterile petri plates and allowed to dry under aseptic conditions. Using a loopful of inoculum, four radial streaks were made on the medium. Plates were incubated at room temperature for 7 days; examined daily for the presence of a dense precipitate around the bacterial growth which is indicative of lipid hydrolysis.

3.7 Identification of biovars

The isolates of *R. solanacearum* collected from different host plants and various locations were categorised into biovars based on their ability to utilize disaccharides (lactose, maltose and cellobiose) and hexose alchohols (mannitol, sorbitol and dulcitol) as a source of carbon and energy (Hayward, 1964; He *et al.*, 1983).

The basal medium used for biovar differentiation was Hayward's semisolid medium (Hayward, 1964). One gram of each carbohydrate was dissolved in 10 ml of sterile distilled water and sterilized by steaming at 100° C for 30 minutes. The solutions were dispersed into six bottles containing 100 ml of molten sterilised basal medium, mixed thoroughly and 5 ml of this was then dispensed into sterile screw capped tubes. There were three replications for each carbohydrate. Thus a total of 18 tubes (6 carbohydrates x 3 replications) were used for each isolate. A control was maintained for each isolate without any carbohydrate.

Bacterial suspension prepared by dispensing single colony in sterile distilled water, adjusted to 10^8cfu/ml, was used for inoculating the tubes. Then 50 µl of the suspension was dispensed into the surface of medium, using a micropipette. Tubes

were incubated at room temperature and examined daily for 14 days for any colour change at surface of the medium.

3.8 Hypersensitivity reaction (HR) test

This test was done to classify the isolates into races. Thirty days old capsicum plants with five to six leaves were used for the HR test. A half ml of standard bacterial inoculum was infiltrated into the intercostal region on the undersurface of a capsicum leaf using plastic disposable syringe with the needle removed. The barrel of the syringe was placed in close apposition to the undersurface of the leaf and pressed gently for facilitating infiltration. Each plant was inoculated on four leaves.

The inoculated plants were examined daily for 10 days after infiltration. The isolates were then grouped into races based on the following criteria (Lozano and Sequeira, 1970).

Race 1 - Dark brown necrosis by 36 h with yellow zone around the edges, by 60 h darkening of vein and veinlets, by 8 to 10 days systemic infection, within 20 days the plants die.

Race 2 - By 10 to 12 h, the leaf area will be water soaked with slight yellowing, by 60 h the affected area becomes papery (brown paper) and will dry up.

Race 3 - Upto 48 h symptomless, by 72 h yellowing of infected area; and limited lesion size.

3.9 Intrinsic antibiotic resistance pattern

Intrinsic antibiotic resistance pattern of bacterial isolates was studied by spotting 10µl of dilute suspension of bacteria in sterile water, on Tryptone Yeast Extract (TY) plates containing various antibiotics at suitable concentrations. Plates were incubated at room temperature and observations taken after 24 and 48 h. TY plate with no antibiotic

served as control. Each treatment was replicated twice. A list of antibiotics along with the concentrations, solvent and source is given below.

Table 3.2 Details of antibiotics used in the study

Antibiotic	Stock mg/ml	Solvent	Working concentration (µg/ml)	Source
Ampicillin	50	Water	50 100	Sigma
Kanamycin	100	Water	50 100	Hi-Media
Streptomycin	75	Water	50 100	Sigma
Tetracycline	50	70% ethanol	25 50	Sigma
Nalidixic acid	100	Water	50 100	Sigma
Gentamycin	10	Water	25 50	Sigma
Chloramphenicol	50	70% ethanol	150 200	Sigma
Rifampicin	75	Water	50 100	Hi-Media
Carbenicillin	100	Water	50 100	Hi-Media

3.10 Molecular characterisation

Isolates of *R. solanacearum* obtained from three different host plants from three locations were characterised by the molecular techniques like plasmid profiling and genetic finger printing using RAPD and RFLP.

Composition of buffers and solutions used for various molecular tests are given in Appendix I.

3.10.1 Plasmid profiling

Plasmid DNA of nine bacterial isolates was extracted using the following protocol. (Santha, 2001)

Procedure:

2 ml LB medium containing proper antibiotic inoculated with a single bacterial colony.

↓

Overnight incubation on a shaker at 200 rpm at 37° C.

↓

Centrifugation at 13000 rpm for 1min.

↓

Supernatant removed carefully.

↓

Cell pellet resuspended in 100 µl resuspension buffer.

↓

Treatment with 100µl lysis buffer and gentle mixing at room temperature.

↓

120 µl of neutralization buffer added, contents mixed gently for 3 min. at room temperature.

↓

Centrifugation at 13000 rpm for 1 min., supernatant transferred to a fresh 1.5 ml centrifuge tube

↓

200 µl isopropanol added and mixed for 1 min.

↓

Centrifugation at 13000 rpm for 1 min. to get DNA pellet.

↓

70% ethanol (500 µl) wash of DNA pellet followed by air-drying.

↓

DNA dissolved in 100 µl sterile water.

10 µl of plasmid DNA sample was mixed with 5 µl of gel loading dye and loaded for electrophoresis.

3.10.1.1 Isolation of megaplasmid

Megaplasmid isolation also was tried using the following protocol (Boominathan and Mahadevan, 1988)

Procedure:

Cells grown in LB medium for 12h

↓

Centrifugation at 10,000g for 10min

↓

Cells suspended in 400µl TES buffer

↓

100µl lysozyme solution added and incubated in ice for 1h

↓

250 µl NaOH-SDS solution and vortexed immediately

↓

Centrifuge tube cap opened, incubated at 70°C for 10min and cooled to room temp.

↓

80 µl phenol:chloroform added, vortexed for 10sec and spun at 5000g for 2min

↓

Upper phase transferred to fresh 1.5ml tube with 50 µl of 3M sodium acetate

↓

500 µl isopropanol added and kept at room temp. for 5min

↓

Centifugation followed by dissolving the pellet in 20 µl TE buffer

10 µl of plasmid DNA sample was mixed with 5 µl of gel loading dye and loaded for electrophoresis.

3.10.1.2 Agarose gel electrophoresis

Preparation of agarose gel:

Gel casting tray was sealed with cellotape to form a mould and set on a horizontal section of the bench after checking the level

↓

One per cent agarose in 1X TAE buffer prepared, the solution boiled and allowed to cool to 42 to 45°C

↓

Ethidium bromide added to a final concentration of 0.5 µg/ml from a stock of 10 mg/ml water, the gel poured to a height of 3 to 5 mm into the casting tray with comb in position and the comb was previously adjusted in such a way that the teeth were 0.5 to 1mm above the plate

↓

Gel was allowed to solidify for 15 to 20 minutes, tape and comb removed; tray was mounted in electrophoresis tank

↓

Electrophoresis buffer (1X TAE) added to the tank to cover the gel to a depth of 1mm.

↓

DNA samples prepared by mixing DNA with gel loading dye in the ratio of 1:1. 20µl was loaded into the slots of gel using a micropipette near the negative terminal

↓

λDNA digested with *Eco*RI and *Hind* III was loaded into the slots on the gel so as to determine the size of the unknown DNA samples

↓

Gel tank was closed, the cathode and anode of the electrophoresis unit were attached to the power supply and a constant voltage of 120 volts was used for the run

↓

Power supply turned off when the loading dye moved about 2/3rd of the gel

The gel was taken from electrophoresis unit and viewed under UV light in a UV transilluminator. Then the gel was documented using Alpha Imager 1200(Alpha Innotech Corporation, USA).

3.10.2 Genetic fingerprinting

RAPD and RFLP were carried out with genomic DNA of bacterial isolates for genetic fingerprinting.

3.10.2.1 Isolation of genomic DNA from bacteria

Genomic DNA was isolated by large prep method (Girija, 1999)

Procedure:

50 ml of overnight bacterial culture prepared in 2 YT medium

↓

40 ml culture centrifuged at 4°C for 10,000 rpm for 10 min

↓

Removal of supernatant and 1% NaCl (10 ml) wash of pellet twice to remove EPS

↓

Pellet dissolved in 8.75 ml TE buffer

↓

Treatment with proteinase K (50 µl) and 10% SDS (1 ml) followed by gentle mixing and incubation at 37°C for 1 h

↓

Chloroform-isoamylalcohol mixture (10 ml) added and mixed for 5 min

↓

Centrifugation at 10,000 rpm at 4°C for 10 min to separate cell debris along with denatured proteins, lipids etc.

↓

Transfer of aqueous phase to a fresh tube

↓

Centrifugation again at 10,000 rpm at 4°C for 10 min after adding and mixing chloroform-isoamyl alcohol mixture (10 ml)

↓

Aqueous phase transferred to a fresh tube, of 1 ml of 5 M sodium acetate and 20 ml isopropanol added and mixed.

↓

DNA thread was spooled out into a fresh tube

↓

70% ethanol wash of DNA pellet at 10,000 rpm for 10 min.

DNA sample (5 µl) was mixed with 5 µl of loading dye and loaded for electrophoresis.

3.10.2.2 Removal of RNA

Procedure:

Pancreatic RNase (RNase A) was used for removing RNA from the DNA extract. A stock of RNase (10 mg/ml) was prepared using 0.01 M sodium acetate at pH 5.2, as solvent. The solution was heated at 100°C for 15 minutes, allowed to cool slowly to room temperature and added 0.1 volume of I M Tris Cl at pH 7.4. The enzyme was stored at –20°C.

Genomic DNA extracted in TE buffer was made up with sterile milli Q water to a volume of 500 µl. RNase was added to a final concentration of 1 µl/100 µl of DNA sample and incubated at 37°C for 1 h in a dry bath. At the end of incubation, an equal volume of phenol: chloroform: isoamyl alcohol mixture (25:24:1, V/V/V) was added, mixed gently and centrifuged at 10,000 rpm for 5 minutes. The aqueous layer was transferred to a fresh eppendorf tube. The process was repeated with chloroform: isoamyl alcohol mixture (24:1, V/V). To the aqueous phase, 2/3 volume of chilled isopropanol was added. DNA was pelletised by centrifugation for 5 minutes at 10,000 rpm at 4°C. Pellet was dried in a vacuum concentrator (Speed Vac Plus, Savant,

USA) for 10 min., redissolved in 50 µl TE buffer and stored at -20^0C until further use. Two µl of this was run on agarose gel to check for complete removal of RNA.

3.10.2.3 Quantification of DNA

After ensuring the quality of DNA in the sample by electrophoresis, 5 to 10 µl of it was diluted to 1.5 ml with sterile milli Q water and absorbance at 260 and 280 nm read using UV visible spectrophotometer (Spectronic R Genesys 5). The purity of DNA was assessed by the ratio OD 260/OD 280. A ratio of 1.8-2.0 indicated pure DNA.

The quantity of DNA in the pure sample was calculated using the formula OD_{260}=1 is equivalent to 50 µg double stranded DNA. Therefore, OD_{260} X 50 gives quantity of DNA in µg/ml.

3.10.2.4 RAPD

RAPD is used as a molecular marker with which the different bacterial strains can be characterised. In this technique, a single short oligonucleotide primers which binds to many different loci is used to amplify random sequences from a template DNA. The amplified DNA samples were electrophoresed as described earlier.

Procedure:

The procedure of Demeke *et al.* (1992) was modified and used for the amplification of bacterial DNA. One cycle included

a. DNA denaturation at 94° C for 1 minute.

b. Annealing of primer to the template DNA at 37.5° C for 1 minute.

c. Primer extension at 72° C for 2 minutes

These were programmed for 45 cycles to get a proper amplification.

The reaction mixture (25 µl) consisted of:

1. 10 X Assay buffer with 15 mM Mg Cl_2 – 5 µl
2. dNTP Mix – 1 µl
3. Taq DNA polymerase – 0.2 µl (0.6 units)
4. Primer – 2 µl (10 pmoles)
5. Template DNA – 1µl (50 ng)
6. Sterile milli Q water to make up to 25 µl

A master mix without primer and template was prepared using the reaction mixture for the required number of reactions. From this master mix, 22 µl was pipetted into each PCR tube, 2 µl of primer and 1 µl of template DNA were added. The reaction mix was overlaid with 25 µl of sterile mineral oil and PCR tubes were loaded in the thermal cycler. The PCR programme was run. The programme took 5 h for completion. The amplified products were electrophoresed on 1.5 per cent agarose gel, the gel was viewed under UV light in transilluminater and then documented using Alpha Imager.

3.10.2.4.1 Screening of random primers for RAPD

A total of 15 decamer primers under different Operon series, viz., OPE, OPF, OPP and OPAH were screened for amplification of genomic DNA extracted from the isolate A3, using the thermal cycle mentioned under 3.10.2.4. From these, ten primers that gave good amplification (three to ten bands) were selected and utilized for further characterisation of nine isolates. The total number of bands along with the number of polymorphic bands obtained in all nine isolates with each of ten primers tried were recorded.

3.10.2.4.3 Analysis of RAPD profiles

The amplification profiles for all the primers were compared with each other and the bands of DNA fragments were scored as present (1) or absent (0) generating the 0, 1 matrices.

The genetic similarity was estimated by computing DICE coefficient using NTSYS pc-2.0 software programme (Dice, 1945; Nei and Li, 1979). The clustering was done and dendrograms were drawn by following unweighted pair group arithmetic mean (UPGMA) routine, using the above programme. Percent polymorphism was also calculated using the following formula,

$$\text{Per cent polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

3.10.2.5 RFLP

Restriction digestion analysis of genomic DNA of nine isolates was carried out using four restriction enzymes, viz., *Hind* III, *Eco*RI, *Bam* HI and *Pst* I according to the method described by Sambrook *et al.* (1982). The enzymes and the assay buffer were bought from Genei, Bangalore.

Procedure:

The following reaction mixture was prepared for genomic DNA digestion with four different restriction enzymes.

Table 3.3 Reaction mixtures for genomic DNA digestion using restriction enzymes

Enzyme	Quantity (µl)					
	DNA	10x buffer	Enzyme	Water	BSA (10 mg/ml)	Final volume
Hind III	20	10	5	65	-	100
*Eco*RI	20	10	5	65	-	100
Bam HI	20	10	5	64	1	100
Pst I	20	10	5	65	-	100

The reaction mix was kept for digestion in dry bath at 37°C overnight. Digested DNA was precipitated by adding 10 µl of 3 M sodium acetate and 100 ml of isopropanol, centrifuged at 10,000 rpm for 10 minutes. DNA pellet was air-dried and dissolved in 20 µl of TE buffer. The whole sample was loaded mixing with 5 µl of gel loading dye. λDNA digested with *Eco*RI and *Hind* III was also loaded as molecular weight marker. Electrophoresis was carried out for 2 h at 100 V and the gel was viewed in a UV transilluminator and documented using Alpha Imager 1200.

3.10.2.6 Southern hybridization

(i) Southern transfer of genomic DNA

The transfer of DNA from agarose gels to nitrocellulose or nylon membranes is referred to as Southern transfer (Southern, 1975; Sambrook *et al.*, 1989).

The gel containing digested DNA samples using *Eco*RI and *Hind* III were used.

Procedure:

Agarose gel containing digested DNA samples was documented for reference and the upper left corner of the gel was cut to mark the orientation.

↓

Immersed in depurination solution for 15 min with intermittent shaking.

↓

Treated with denaturation solution for 45 min followed by neutralization solution for 30 min with slight shaking.

↓

Washed in 1 X SSC for 30 min.

↓

A capillary Southern transfer assembly was set up. It consisted of 500 ml of 1X SSC solution taken in a tray above which a thick glass plate was placed. A strip of Whatman filter paper was placed over the glass plate. The filter paper was soaked in 1X SSC and air bubbles between the glass plate and the filter paper were removed.

↓

The gel was placed on top of filter paper, and moistened with 1 X SSC.

↓

A nitrocellulose membrane exactly the same size of the gel was placed over the gel. The slots were marked on the membrane using a pencil.

↓

Three sheets of Whatman paper and a stack of paper towels (10 cm high) were placed on top of the nitrocellulose membrane.

↓

A glass plate was placed on the stack and a 1 kg weight was kept and allowed the transfer to proceed overnight.

↓

Membrane was rinsed in 1 X SSC and air dried on a piece of Whatman paper.

↓

Baked for 2 h at 80°C under vacuum.

(ii) Prehybridization

Procedure:

The membrane was prehybridized at 65°C for 4 h with prehybridization solution at the rate of 0.1 ml/cm^2 of the membrane in glass tubes in hybridization oven.

(iii) Extraction of probe DNA from agarose gel

Materials required

Gel fragment containing DNA of interest. Band specific to A1, A2 and A3 obtained on PCR with OPF8 was used as the probe.

Clean Genei kit (Genei, Bangalore) consisting of

A - Glass solution

B - Sodium iodide solution

C - Wash buffer

Sterile milli Q water

Microfuge

Procedure:

DNA bands excised with a sharp blade and the gel pieces taken in a microfuge tube

↓

2.5 volume of sodium iodide solution was added.

↓

Gel pieces were solubilised by incubating at 45-55°C for 2 to 3 minutes.

↓

15 µl of glass solution added to the solubilised sample, contents mixed thoroughly and kept it at room temperature for 5 minutes with occasional mixing.

↓

Centrifugation at 12,000 rpm for 30 seconds, supernatant discarded.

↓

200 µl wash buffer added, vortexed and then centrifuged at 12,000 rpm for 30 seconds, discarding the supernatant.

↓

The above step was repeated and tube was left at 37°C for 10 min. to remove the traces of wash buffer.

↓

30 µl sterile milli Q water was added, and pellet resuspended and incubated at 45-55°C for 5 min.

↓

Centrifuged at 12,000 rpm for 30 sec to collect supernatant in fresh tube.

↓

20 µl of water was added to the pellet, supernatant was collected and pooled. Glass powder was removed by centrifugation for 1 min. and DNA was checked on gel.

(iv) Labelling of the DNA probe

The probe DNA was labelled using random primer labelling kit purchased from Genei, Bangalore.

Materials required

1. Radioisotope – (α ^{32}p) dCTP (specific activity >3000 Ci/m mole; 10 u Ci/µl)
2. Klenow fragment (3 units/µl)
3. Random hexanucleotides (100 ng//µl)
4. 10 x labelling buffer
5. d NTPs (2.5 mM stock from 10 mM dATP, dGTP and dTTP)
6. Nuclease free water
7. Dithiothreitol (DTT) (20Mm)

Protocol:

10 µl of probe DNA taken in an autoclaved eppendorf tube

↓

9 µl of autoclaved MilliQ water added

↓

The contents boiled for 5 min. in boiling water bath, chilled on ice for 5 min.

↓

Centrifuged at 8000 rpm for 30 seconds.

↓

2.5 µl of 10 x labelling buffer, 1 µl of random primer, and 2.5 µl of DTT solution added.

↓

2 µl of dNTP mix added.

↓

3 µl of [α ^{32}p] dCTP, 3 µl of autoclaved milliQ water and 1 µl of Klenow were added,contents mixed.

↓

Incubated at room temperature for 2 h.

(v) Hybridization

The radioactive labelled DNA probe which was in double stranded form was denatured in a boiling water bath and added to the membrane in prehybridization solution. Hybridization was allowed to proceed at 65°C overnight.

(vi) Autoradiography

The hybridization membrane was washed for 15 min. each in 2 X SSC, 2 X SSC + 0.1% SDS and finally 1 X SSC at room temperature. Membrane was dried, covered with a cling film, placed in a lead cassette and exposed to an X-ray film (Konica) for three to five days at –20°C.

Processing of the X-ray film

This was carried out in a dark room with safety lights having red filters.

The film was washed sequentially in following solutions.

X-ray developer for 3 min.

↓

Stop solution (1% acetic acid) for 30 sec.

↓

Distilled water for 1 min.

↓

Fixer solution for 3 min.

↓

Running water for 20 min

Film was dried at room temperature, and then observed autoradiogram.

3.11 Assessment of the role of plasmid in EPS production

3.11.1 Plasmid profile of mucoid and non-mucoid colonies

When bacterial suspensions from stock cultures of V2, V3 and K3 were streaked on TZC medium, only the typical fluidal white mucoid colonies with pink centres formed initially. After three to five days, mutants could be detected in the form of non-mucoid, deep red colonies in the same petriplate. Hence the plasmid profiles of both mucoid and non-mucoid colonies were compared to assess the role of plasmid in EPS production.

Both mucoid and non-mucoid colonies selected from the same petriplate of V3 were inoculated separately into 5 ml of LB medium and kept in shaker overnight. The plasmid profiles of both these colonies were analysed by isolating plasmid DNA using the protocol of plasmid DNA isolation given under 3.10.1 and then electrophoresis was carried out on 1per cent agarose gel.

3.11.2 Plasmid curing

Curing of plasmids is the process of elimination of plasmid from the bacterium and getting it free of the plasmid coded characters. Generally curing agents fall into 3 categories i.e., U.V. irradiation, intercalating dyes and antibiotics and high temperature.

Curing of plasmids using high temperature was tried to check the loss of EPS production trait with the loss of plasmid. A 12 h bacterial culture was prepared in 5 ml of LB medium and incubated at temperatures varying from 40 to 45°C for 12, 36 and 48 h. Then 1 ml of it was diluted with 5 ml of sterile distilled water, spread plated on TZC medium and incubated for 48 h at room temperature for the development of non-mucoid colonies. Five single colonies were picked up at random from each plate and plasmid preparation was done as per 3.10.1.

Results

4. RESULTS

The results of the study on " Molecular characterisation of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* causing bacterial wilt in solanaceous vegetables " undertaken at Department of Plant Pathology and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara are presented below.

4.1 Isolation and maintenance of the pathogen

Bacterial wilt affected brinjal, chilli and tomato plants were collected from three different locations viz., Vellanikkara, Kumarakom and Ambalavayal. The bacteria were isolated on TZC medium. Creamy white colonies with pink centre, characteristic of *R. solanacearum*, were selected and purified by repeated streaking on TZC medium. Koch's postulates were proved with each isolate on their respective host plants. Five typical single colonies suspended in 5 ml sterile distilled water in screwcapped vials and stored at room temperature, were found to maintain virulence.The isolates were tested on TZC medium once in two months for virulence.

4.2 Standardisation of the inoculation technique

Inoculation technique was standardized in brinjal, chilli and tomato using incubation period (IP) and per cent wilt incidence (PWI). The data on IP and PWI one week after inoculation are given in Table 4.1

In brinjal, a 100 per cent wilt incidence was noticed in root dipping, stem puncturing and leaf clipping one week after inoculation. But the wilt symptom was first evident in plants inoculated by root dipping, on 5^{th} day after inoculation. Hence it was considered as the best method of inoculation in brinjal (Plate 1a).

In chilli, the wilt symptoms were first observed on 6^{th} day of inoculation in all methods of inoculation except in soil drenching. However, a 100 per cent wilt

Table 4.1 Standardisation of inoculation technique in brinjal, chilli and tomato based on PWI and IP.

Methods of inoculation	Brinjal		Chilli		Tomato	
	PWI	IP (days)	PWI	IP (days)	PWI	IP (days)
SP	100.00	6	33.33	6	100.00	6
LC	100.00	6	100.00	6	100.00	5
SD	0	11	33.33	7	33.33	7
SD+W	0	7	66.67	6	66.67	7
RD	100.00	5	33.33	6	33.33	6

PWI: Per cent wilt incidence IP: Incubation period

SP: Stem Puncturing SD+W: Soil Drenching with Wounding

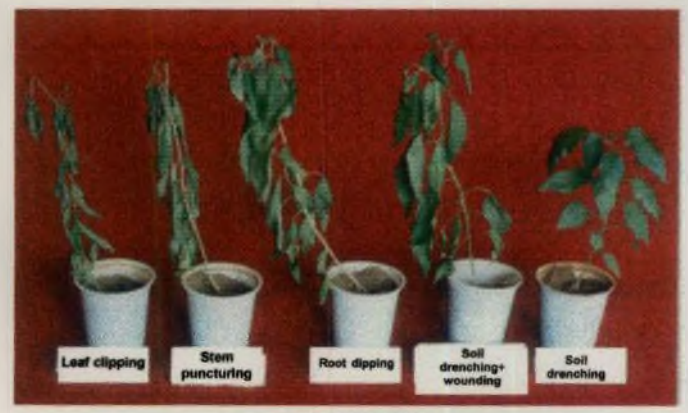
LC: Leaf Clipping RD: Root Dipping

SD: Soil Drenching

Plate - 1

Effect of different methods of inoculation on bacterial wilt incidence

a - Brinjal



b - Chilli

c - Tomato

incidence was shown by plants inoculated using leaf clipping on 7th day after inoculation.Soil drenching with wounding was the next best method having 66.67 per cent wilt incidence with an incubation period of 6 days (Plate 1b).

In tomato, both leaf clipping and stem puncturing recorded 100 per cent wilt incidence on 7th day after inoculation. But the minimum incubation period of 5 days was shown by leaf clipping, hence considered as the best method (Plate1c).

4.3 Comparison of virulence and aggressiveness of *R. solanacearum* isolates

The virulence and aggressiveness of the isolates were compared by inoculating on the respective host plants under artificial conditions.

4.3.1 Virulence

Virulence of the isolates measured based on BWI calculated on 5, 10 and 15 days after inoculation is given in Table 4.5.

Among the brinjal isolates, K1 was found the most virulent since even on 5th day after inoculation, it had BWI of 48.3 per cent whereas V1 and A1 recorded only 10 per cent wilt incidence. The least virulent isolate of brinjal was A1since it had only 25 per cent BWI on 15th day after inoculation. The disease progress of the isolates of brinjal is given in Table 4.2. The variation in virulence pattern of the brinjal isolates is shown in Fig. 1a.

Fig. 1b shows difference in the virulence pattern of chilli isolates of *R. solanacearum*. K2 among chilli isolates recorded 68.3 per cent BWI even on 5th day after inoculation. The disease progress was so fast that by 7th day of inoculation, it attained BWI of 100%. Among the chilli isolates, A2 recorded the minimum BWI of 21.66 per cent on 15th day after inoculation. Table 4.3 shows the comparison of disease progress of the three chilli isolates. Comparison could be done only for eight days, since plants inoculated with K2 completely wilted within seven days.

Table 4.2. Pathogenicity rating of brinjal isolates from different locations on brinjal (disease score)

Isolate	Days after inoculation									
	3	4	5	6	7	8	9	10	11	12
V1	0 (0.707)	0.33 (0.880)	0.50 (0.966)	0.50 (0.966)	0.67 (1.052)	0.67 (1.052)	1.50 (1.342)	1.50 (1.342)	1.67 (1.406)	1.92 (1.495)
K1	1.58 (1.329)	2.17 (1.540)	2.42 (1.625)	2.42 (1.625)	2.83 (1.760)	2.83 (1.760)	3.25 (1.872)	3.25 (1.872)	3.75 (2.013)	3.75 (2.013)
A1	0.50 (0.966)	0.50 (0.966)	0.50 (0.966)	0.50 (0.966)	0.92 (1.168)	0.92 (1.168)	0.92 (1.168)	0.92 (1.168)	0.92 (1.168)	1.25 (1.281)
$CD_{(0.05)}$	0.315	0.315	0.315	0.287	0.287	0.287	0.3445	0.3445	0.3445	0.3445

Table 4.3. Pathogenicity rating of chilli isolates from different locations on chilli (disease score)

Isolate	Days after inoculation					
	3	4	5	6	7	8
V2	0 (0.707)	0 (0.707)	0.92 (1.1238)	1.67 (1.355)	2.25 (1.556)	2.25 (1.556)
K2	1.67 (1.413)	1.67 (1.413)	3.42 (1.958)	4.33 (2.189)	5.00 (2.345)	5.00 (2.345)
A2	0.25 (0.837)	0.25 (0.837)	0.25 (0.837)	0.25 (0.837)	0.92 (1.141)	0.92 (1.141)
$CD_{(0.05)}$	0.2296	0.2296	0.2583	0.3157	0.3445	0.3445

Table 4.4. Pathogenicity rating of tomato isolates from different locations on tomato (disease score)

Isolate	Days after inoculation									
	3	4	5	6	7	8	9	10	11	12
V3	0.42 (0.909)	0.42 (0.909)	1.62 (1.230)	1.17 (1.230)	2.42 (1.653)	2.42 (1.653)	3.4 (1.965)	3.42 (1.965)	4.75 (2.289)	4.75 (2.289)
K3	2.83 (1.793)	2.83 (1.793)	3.92 (2.088)	3.92 (2.088)	4.08 (2.131)	4.08 (2.131)	4.08 (2.131)	4.08 (2.131)	4.92 (2.327)	4.92 (2.327)
A3	0 (0.707)	0 (0.707)	0.92 (1.062)	0.92 (1.062)	1.33 (1.160)	1.33 (1.160)	1.33 (1.160)	1.33 (1.160)	1.58 (1.276)	1.58 (1.276)
$CD_{(0.05)}$	0.2296	0.2296	0.3445	0.3445	0.4306	0.4306	0.3732	0.3732	0.3445	0.3445

Figures in parenthesis indicate $\sqrt{(x + 1/2)}$ transformed values

V : Vellanikkara K : Kumarakom A : Ambalavayal
1 : Brinjal 2 : Chilli 3 : Tomato

Similarly in the case of tomato, K3 was found to be the most virulent one and A3, the least (Fig. 1c). The average disease score of tomato isolates recorded 12 days after inoculation is presented in Table 4.4.

The virulence pattern of isolates of *R. solanacearum* based on BWI is shown in Fig. 2a.

4.3.2 Aggressiveness

Aggressiveness of all the isolates was calculated based on incubation period (IP) and latent period (LP_{50}) in days and the data are presented in Table 4.6. Among the brinjal isolates, the most aggressive one was K1 with minimum IP (2.25) and LP_{50} (3.0). Similarly, in the case of chilli and tomato, Kumarakom isolates showed more aggressiveness compared to those from Vellanikkara and Ambalavayal. V1 from Vellanikkara, A2 and A3 from Ambalavayal were found less aggressive (Fig. 2b and 2c).

4.4 Cross inoculation of the isolates

The data of cross inoculation studies of *R. solanacearum* isolates based on BWI calculated 10 days after inoculation and IP are presented in Table 4.7.

In cross inoculation studies, all Vellanikkara and Kumarakom isolates were found cross inoculable with each other.

Among Vellanikkara isolates, V2 was more virulent on brinjal compared to V3. V2 developed wilt symptoms in brinjal on 6^{th} day after inoculation whereas V3 took 7.25 days to produce symptoms on brinjal. V3 was more virulent on chilli compared toV1. On tomato, V1 showed high BWI when compared to V2.

In the cross inoculation studies of Kumarakom isolates, it was found that, on brinjal, K3 was more virulent compared to K2. K1 produced a maximum BWI of 100 per cent on chilli and tomato with an incubation period of only 3.5 days. K2 was also highly virulent on tomato with BWI of 100 per cent.

Fig. 1a. Pathogenicity rating of brinjal isolates of *R. solanacearum*

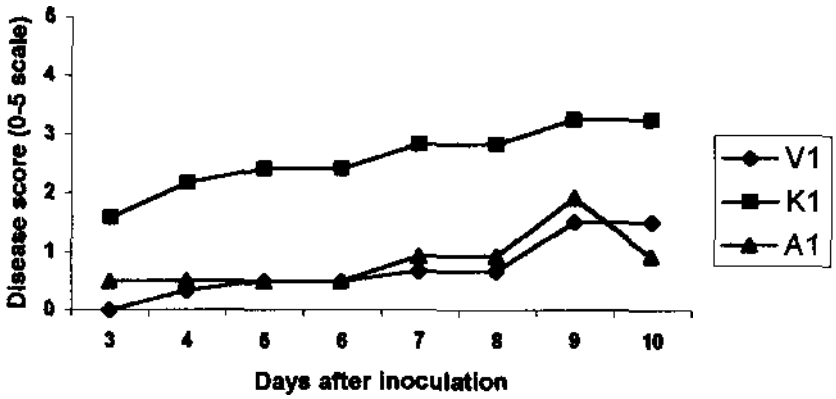


Fig. 1b. Pathogenicity rating of chilli isolates of *R. solanacearum*

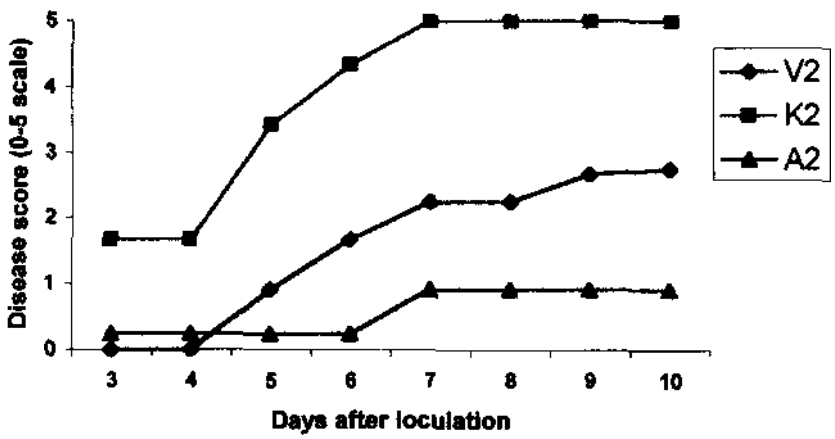


Fig. 1c. Pathogenicity rating of tomato isolates of *R. solanacearum*

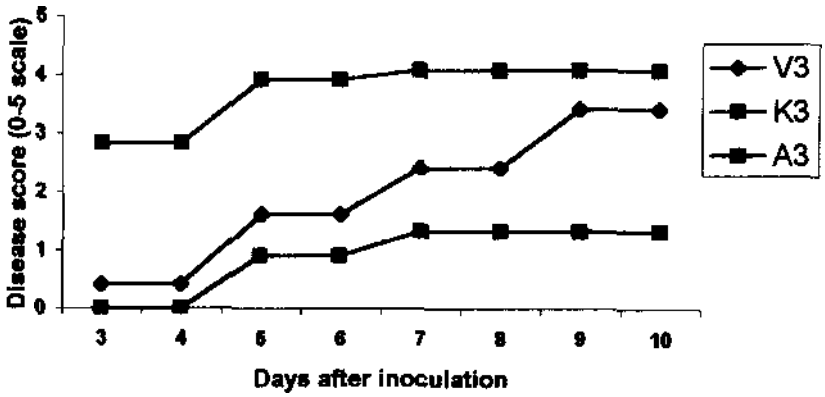


Table 4.5. Virulence of *R.solanacearum* isolates on respective host plants based on BWI (%)

Host plant	Bacterial wilt index (%)								
	Vellanikkara			Kumarakom			Ambalavayal		
	5 DAI	10DAI	15DAI	5 DAI	10DAI	15DAI	5 DAI	10DAI	15DAI
Brinjal	10.00 (W)	18.30 (W)	48.30 (M)	48.30 (M)	50.00 (M)	81.66 (H)	10.00 (W)	16.67 (W)	25.00 (W)
Chilli	18.30 (W)	56.67 (M)	73.30 (H)	68.30 (H)	100.00 (H)	100.00 (H)	5.00 (W)	18.30 (W)	21.66 (W)
Tomato	23.30 (W)	78.30 (H)	100.00 (H)	78.30 (H)	92.50 (H)	100.00 (H)	18.30 (W)	26.67 (W)	38.30 (W)

DAI : days after inoculation
H : Highly virulent (>65% BWI) M : Moderately virulent (40-65% BWI)
W : Weakly virulent (<40% BWI)

Table 4.6. Aggressiveness of *R.solancearum* isolates based on IP and LP_{50} (days)

Host plant	Vellanikkara		Kumarakom		Ambalavayal	
	IP	LP_{50}	IP	LP_{50}	IP	LP_{50}
Brinjal	5.25 (2.362)	6.5 (2.616)	2.25 (1.654)	3.00 (1.851)	3.000 (1.871)	6.25 (2.563)
Chilli	4.25 (2.177)	6.25 (2.560)	3.00 (1.871)	3.00 (1.871)	4.25 (2.132)	8.00 (2.915)
Tomato	3.50 (1.989)	4.5 (2.222)	2.00 (1.581)	2.25 (1.654)	11.00 (3.264)	14.00 (3.710)
CD(0.05)	0.6378	0.6668	0.6938	0.7562	0.7982	0.7982

IP : Incubation period LP_{50} : Number of days for 50% wilting of plants

Figures in parenthesis indicate $\sqrt{(x + 1/2)}$ transformed values

Fig.2a. Virulence of *R. solanacearum* isolates based on BWI

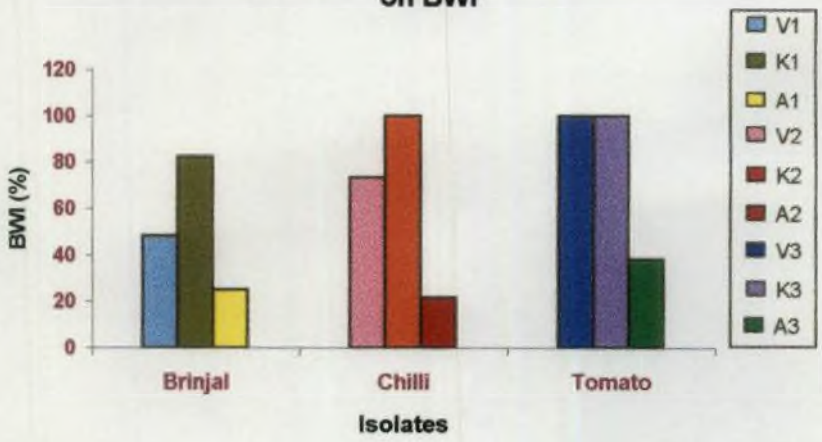


Fig. 2b. Aggressiveness of *R. solanacearum* isolates based on IP

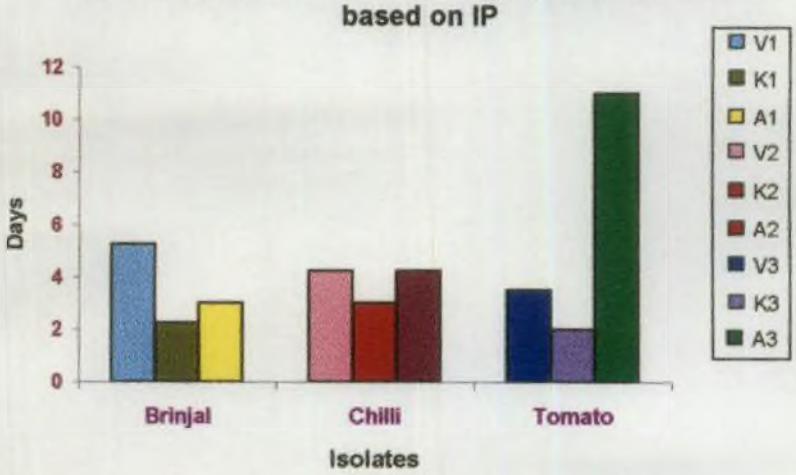


Fig.2c. Aggressiveness of *R. solanacearum* isolates based on LP_{50}

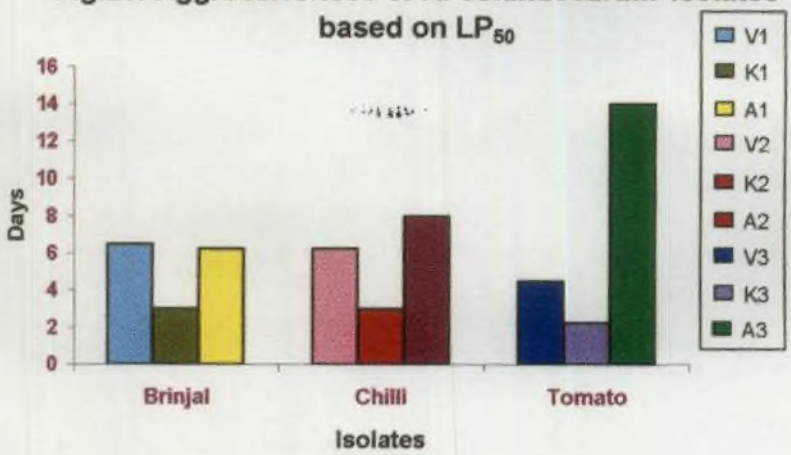


Table 4.7. BWI and IP of *R. solancearum* isolates in cross inoculation studies

Host plant / Isolate	Brinjal		Chilli		Tomato	
	BWI (%)	IP (days)	BWI (%)	IP (days)	BWI (%)	IP (days)
V1	18.3 (W)	5.25	40.00 (M)	7.50	90.0 (H)	4.00
V2	30.0 (W)	6.00	56.67 (M)	4.25	87.5 (H)	3.25
V3	27.5 (W)	7.25	45.00 (M)	6.50	78.3 (H)	3.50
K1	50.00 (M)	2.25	100.00 (H)	3.50	100.00 (H)	3.50
K2	15.00 (W)	7.75	100.00 (H)	3.00	100.00 (H)	3.00
K3	40.00 (M)	4.5	50.00 (M)	4.75	92.5 (H)	2.00
A1	16.67 (W)	3.00	5.00 (W)	13.00	0 (A)	-
A2	0 (A)	-	18.30 (W)	4.25	0 (A)	-
A3	0 (A)	-	10.00 (W)	12.00	26.67 (W)	11.00

V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

H : Highly virulent (>65% BWI)
M: Moderately virulent (40-65% BWI)
- : No symptom development

W : Weakly virulent (<40% BWI)
A : Avirulent (BWI-0)

Ambalavayal isolates were not cross inoculable. Very low BWI of 5 and 10 per cent could be noticed on chilli when cross inoculated with A1 and A3 respectively 10 days after inoculation.

4.5 Characterisation of *Ralstonia solanacearum* isolates

4.5.1 Morphological characterisation

All isolates were Gram negative, rod shaped and motile when viewed under microscope.

4.5.2 Cultural characterisation

Results of studies on the characterisation of different isolates of *R. solanacearum* from diseased tomato, brinjal and chilli wilted plants collected from different locations showed slight variations in cultural characteristics (Table 4.8). All isolates formed circular colonies with entire margin. Elevation varied from flat to slightly raised. In the case of Vellanikkara isolates, all were having creamy white circular colonies with light pink centre. V2 was highly fluidal compared to the other two and V3 was less slimy (Plate 2a). Kumarakom isolates also formed creamy white colonies with light pink centre. K1 and K2 were highly fluidal compared to K3 (Plate 2b). Isolates obtained from Ambalavayal also produced creamy white colonies, but with more reddish pink centre. The fluidity was high in A2 compared to A1 and A3. The colonies of A1 and A2 were seen slightly raised, but that of A3 was flat (Plate 2c). The development of non-mucoid mutant colonies was noticed in certain isolates after long-term storage in sterile distilled water due to loss of virulence (Plate 2d).

4.5.3 Biochemical characterisation

The isolates were biochemically characterised by nine different tests. The data are presented in Table 4.9.

4.5.3.1 Solubility in 3per cent KOH

All isolates formed viscous strands with 3per cent KOH.

Table 4.8. Cultural characteristics of *R. solancearum* isolates on TZC medium (after 48 h)

Isolates	Colony characters					
	Size	Pigmentation	Form	Margin	Elevation	Fluidity
V1	Pinpoint to small	Creamy white with pink centre	Circular	Entire	Flat	Low
V2	Small to moderate	Creamy white with pink centre -	Circular	Entire	Slightly raised	High
V3	Pinpoint	Creamy white with pink centre -	Circular	Entire	Flat	Very low
K1	Small to moderate	Creamy white with pink centre -	Circular	Entire	Slightly raised	High
K2	Small to moderate	Creamy white with pink centre -	Circular	Entire	Slightly raised	High
K3	Pinpoint	Creamy white with pink centre	Circular	Entire	Flat	Low
A1	Pinpoint	Creamy white with reddish pink centre	Circular	Entire	Slightly raised	High
A2	Small to moderate	Creamy white with reddish pink centre	Circular	Entire	Slightly raised	Very high
A3	Pinpoint	Creamy white with reddish pink centre	Circular	Entire	Flat	High

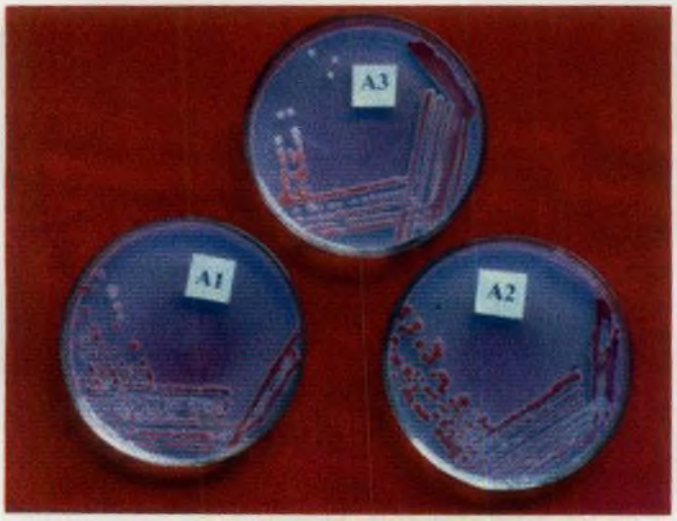
V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

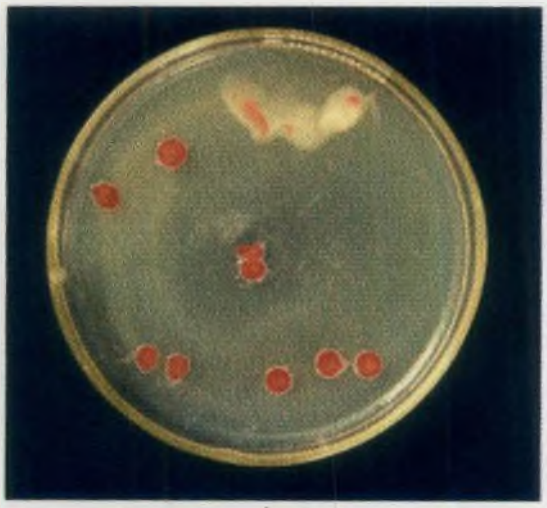
Plate - 2
***R. solanacearum* isolates from different locations on TZC medium**

a - Vellanikkara

b - Kumarakom



c - Ambalavayal



d - Mutants of *R. solanacearum* on TZC medium

4.5.3.2 Kovacs' oxidase test

Purple colour developed with oxidase discs by all isolates. Time taken for development of purple colour varied among the isolates (Table 4.10). Vellanikkara isolates developed purple colour rapidly (within 10 seconds). Kumarakom isolates were delayed positive. Among Ambalavayal isolates, A3 took about a minute for purple colour development whereas other two developed colour rapidly.

4.5.3.3 Catalase test

The isolates were all catalase positive indicating the presence of catalase enzyme in the isolates. Vellanikkara and Ambalavayal isolates produced large number of gas bubbles within a few seconds on mixing them with 3 per cent H_2O_2. However, among Kumarakom isolates, K1 and K2 showed delayed reaction with less number of bubbles.

4.5.3.4 Fluorescent pigment

None of the nine isolates produced fluorescent pigment on King's B Medium containing 1 per cent tyrosine. Instead, all the isolates produced brown diffusible melanin pigment on it.

4.5.3.5 Levan from sucrose

Isolates from Vellanikkara and Kumarakom did not produce levan on King's B medium containing 5 per cent sucrose. All the three Ambalavayal isolates produced slightly raised convex colonies, indicative of levan production.

4.5.3.6 Arginine dihydrolase reaction

None of the isolates produced ammonia from arginine.

Table 4.9. Biochemical characteristics of different isolates of *R.solancearum*.

Sl. No.	Biochemical tests	Isolates								
		V1	V2	V3	K1	K2	K3	A1	A2	A3
1	Solubility in 3% KOH	+	+	+	+	+	+	+	+	+
2	Kovacs' oxidase	+	+	+	+	+	+	+	+	+
3	Fluorescent pigment production	–	–	–	–	–	–	–	–	–
4	Levan from sucrose	–	–	–	–	–	–	±	±	±
5	Glucose oxidation	+	+	+	+	+	+	+	+	+
6	Catalase	+	+	+	+	+	+	+	+	+
7	Arginine dihydrolase reaction	–	–	–	–	–	–	–	–	–
8	Denitrification	+	+	+	–	–	–	–	–	–
9	Lipase activity	+	+	+	+	+	+	+	+	+

+ = Positive – = Negative ± = Slightly positive

V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

Table 4.10. Reaction of different isolates of *R.solancearum* to Kovacs' oxidase reagent

Isolate	Time taken for purple colour development	Inference
V1	< 10 seconds	Positive
V2	< 10 seconds	Positive
V3	< 10 seconds	Positive
K1	1 minute	Delayed positive
K2	1 minute	Delayed positive
K3	1 minute	Delayed positive
A1	<10 seconds	Positive
A2	<10 seconds	Positive
A3	<10 seconds	Delayed positive

V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

4.5.3.7 Glucose oxidation

Glucose was oxidised in both sealed and unsealed tubes by all isolates. In sealed tubes, gas production resulted in breaking of the agar seal from the lower basal medium after 24 h (Plate 3a).

4.5.3.8 Denitrification

VMG and VMS media were used for denitrification test. Vellanikkara isolates denitrified in the presence of both glycerol and succinate (Plate 3b and 3c) whereas Kumarakom and Ambalavayal isolates did not produce gas from nitrate in the presence of neither glycerol nor succinate.

4.5.3.9 Lipase activity

White dense precipitate could be seen around all the isolates streaked on Tween 80 agar, indicating positive reaction.

4.5.4 Identification of biovars

Isolates of *R. solanacearum* collected from three locations differed in their ability to oxidise various hexose alcohols and disaccharides. Time required for utilisation of these carbohydrates also varied among the different isolates.

V1 and V2 from Vellanikkara utilized all carbohydrates whereas V3 was negative for dulcitol (Table 4.11). V1 utilised all carbohydrates by 7 days, while V2 took only 3 days for utilisation. Evenafter 14 days, V3 could not utilize dulcitol. So V1 and V2 collected from Vellanikkara belonged to biovar III whereas V3 belonged to biovar IIIA.

Among Kumarakom isolates, K3 utilized all carbohydrates by one day whereas K2 took 2 days. Hence these two isolates belonged to biovar III. K2 could not utilize dulcitol evenafter 14 days and hence it belonged to biovar IIIA (Table 4.12).

Table 4.11. Differentiation of Vellanikkara isolates of *R.solancearum* into biovars

Period (days)	Isolates	Carbohydrate						Biovar
		Lactose	Maltose	Cellobiose	Mannitol	Sorbitol	Dulcitol	
1	V1	–	±	–	–	–	–	
	V2	±	∓	–	–	–	–	
	V3	∓	±	±	±	±	–	
2	V1	+	+	–	–	–	–	
	V2	±	±	–	–	–	–	
	V3	∓	+	+	+	+	–	
3	V1	∓	+	–	+	∓	∓	
	V2	±	+	∓	∓	∓	∓	
	V3	+	+	+	+	+	–	III
4	V1	+	+	–	∓	∓	+	
	V2	+	+	±	±	±	±	
	V3	+	+	+	+	+	–	
7	V1	+	+	∓	±	±	±	III
	V2	+	+	+	+	+	+	
	V3	+	+	+	+	+	–	
14	V1	+	+	+	+	+	+	III
	V2	+	+	+	+	+	+	III
	V3	+	+	+	+	+	–	IIIA

+ : Complete colour change
– : No colour change

∓ :slight colour change
± : partial colour change

V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

Table 4.12. Differentiation of Kumarakom isolates of *R.solancearum* into biovars

Period (days)	Isolates	Carbohydrate						Biovar
		Lactose	Maltose	Cellobiose	Mannitol	Sorbitol	Dulcitol	
1	K1	–	∓	∓	∓	∓	–	
	K2	∓	±	±	±	±	–	
	K3	∓	∓	∓	∓	∓	∓	III
2	K1	+	±	±	±	±	∓	III
	K2	±	+	±	+	+	–	
	K3	+	∓	∓	∓	∓	∓	
3	K1	±	+	±	±	±	±	
	K2	+	+	±	+	+	–	
	K3	±	+	∓	±	±	±	
4	K1	±	+	±	±	±	±	
	K2	+	+	±	+	+	–	
	K3	±	+	∓	±	±	±	
7	K1	+	+	±	±	±	±	
	K2	+	+	±	+	+	–	
	K3	+	+	∓	±	±	±	
14	K1	+	+	+	+	+	+	III
	K2	+	+	+	+	+	–	IIIA
	K3	+	+	+	+	+	+	III

+ : Complete colour change
– : No colour change

∓ :slight colour change
± : partial colour change

V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

Table 4.13. Differentiation of Ambalavayal isolates of *R.solancearum.* into biovars

Period (days)	Isolates	Lactose	Maltose	Cellobiose	Mannitol	Sorbitol	Dulcitol	Biovar
1	A1	–	∓	±	±	±	±	III
	A2	–	∓	–	±	–	–	
	A3	∓	∓	±	±	∓	±	
2	A1	∓	+	+	+	±	±	III
	A2	∓	+	∓	+	∓	–	
	A3	±	+	+	+	±	±	
3	A1	±	+	+	+	+	+	
	A2	∓	+	±	+	±	–	
	A3	+	+	+	+	+	±	
4	A1	±	+	+	+	+	+	
	A2	±	+	+	+	±	–	
	A3	+	+	+	+	+	+	
7	A1	+	+	+	+	+	+	
	A2	+	+	+	+	+	–	
	A3	+	+	+	+	+	+	
14	A1	+	+	+	+	+	+	III
	A2	+	+	+	+	+	–	IIIA
	A3	+	+	+	+	+	+	III

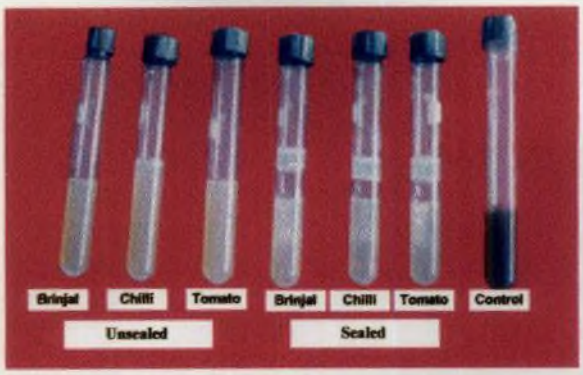
+ : Complete colour change
– : No colour change
∓ :slight colour change
± : partial colour change

V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

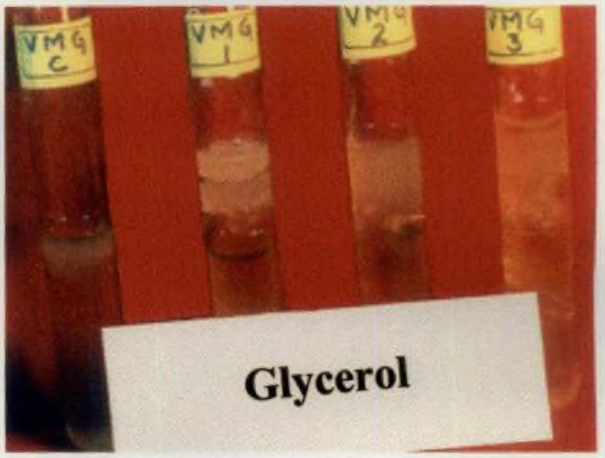
Plate - 3 Biochemical Characteristics

a - Oxidation of Glucose

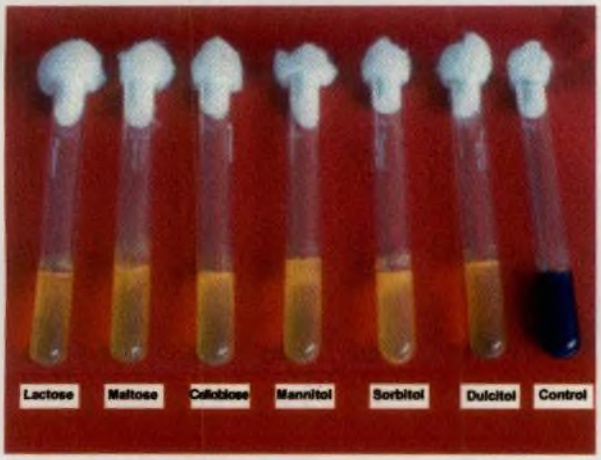


b. Denitrification using Succinate

c. Denitrification using Glycerol



d. Biovar III



e. Biovar III A

f. Gas production from carbohydrates

Ambalavayal isolates also showed differences in utilization of sugars. A3and A1 utilised the carbohydrates by one day and 2 days respectively and hence these were grouped under biovar III. A2 belonged to biovar IIIA since it could not utilize dulcitol (Table 4.13).

The differentiation of all isolates of *R. solanacearum* into biovars is given in Table 4.14.

4.5.5 Hypersensitivity Reaction (HR) test

The hypersensitivity reaction of different isolates of *R. solanacearum* on *Capsicum* leaves is given in Table 4.15.

Tomato isolates collected from Vellanikkara (V3) and Kumarakom (K3) developed water soaking after 1 to 2 days of infiltration on capsicum leaves (Plate 4a). These spots later turned to typical necrotic lesion with yellow halo by 3 to 4 days characteristic of race 1 (Plate 4b). Brinjal and chilli isolates of Vellanikkara (V1 and V2) and Kumarakom (K1 and K2) caused wilting of capsicum plants within a period of 10 days without producing necrotic lesions, also characteristic of race 1. In the case of Ambalavayal isolates, though initially water soaking developed, no necrotic lesions formed. The infiltrated area turned yellow by 2 to 4 days. Later, yellowing of whole leaves was observed within 10 days, characteristic of race 3 (Plate 4c).

4.6 Intrinsic antibiotic resistance pattern

The antibiotic sensitivity pattern of the nine isolates was identified. All nine isolates were resistant to ampicillin at both concentrations, 50 and 100 µg/ml and sensitive to chloramphenicol at 150 and 200 µg/ml and nalidixic acid at 100 µg/ml. These isolates differed from one another in susceptibility to other antibiotics tested at two different concentrations (Table 4.16).

Plate - 4
Hypersensitive reaction on *Capsicum*

⇦ a . Water soaking - Race 1

b . Necrotic lesion with yellow halo - Race 1 ⇨

⇦ c. Yellowing of leaves - Race 3

Table 4.14. Differentiation of isolates of *R. solanacearum* into biovars.

Isolates	Carbohydrates						Biovar
	Lactose	Maltose	Cellobiose	Mannitol	Sorbitol	Dulcitol	
V1	+	+	+	+	+	+	III
V2	+	+	+	+	+	+	III
V3	+	+	+	+	+	–	IIIA
K1	+	+	+	+	+	+	III
K2	+	+	+	+	+	–	IIIA
K3	+	+	+	+	+	+	III
A1	+	+	+	+	+	+	III
A2	+	+	+	+	+	–	IIIA
A3	+	+	+	+	+	+	III

Table 4.15 . Hypersensitive reaction on *Capsicum* with isolates of *R.solancearum*

Isolate	Days after infiltration				Race
	1	2	3 to 4	10	
V1	--	Water soaking	--	Wilting	1
V2	--	Water soaking	--	Wilting	1
V3	Water soaking	Brown necrotic lesion	Lesion with yellow halo	Wilting	1
K1	--	Water soaking	--	Wilting	1
K2	Water soaking	Water soaking	--	Wilting	1
K3	Water soaking	Brown necrotic lesion	Lesion with yellow halo	Wilting	1
A1	Water soaking	Yellowing of infiltrated area	Yellowing of leaves	Yellowing of whole plant	3
A2	Water soaking	Yellowing of infiltrated area	Yellowing of leaves	Yellowing of whole plant	3
A3	Water soaking	Yellowing of infiltrated area	Yellowing of leaves	Yellowing of whole plant	3

V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

-- : No symptom

Table 4.16. Antibiotic sensitivity pattern of different isolates of *R.solancearum*

Antibiotic	Concentration (µg/ml)	V1	V2	V3	K1	K2	K3	A1	A2	A3
Ampicillin	50	+	+	+	+	+	+	+	+	+
	100	+	+	+	+	+	+	+	+	+
Rifampicin	50	+	±	+	±	±	–	+	+	+
	100	+	–	+	–	–	–	±	±	+
Carbenicillin.	50	+	+	+	+	+	–	+	+	+
	100	+	+	+	+	±	–	+	+	+
Kanamycin	50	+	–	+	–	±	+	+	+	±
	100	+	–	+	–	±	+	+	+	±
Gentamycin	25	–	–	+	–	–	–	+	±	±
	50	–	–	±	–	–	–	±	±	±
Tetracycline	25	–	–	±	–	–	+	+	+	+
	50	–	–	–	–	–	±	–	–	–
Nalidixic acid	50	–	–	±	–	–	±	–	–	–
	100	–	–	–	–	–	–	–	–	–
Chloramphenicol	150	–	–	–	–	–	–	–	–	–
	200	–	–	–	–	–	–	–	–	–
Streptomycin	50	±	–	+	–	–	+	+	+	+
	100	–	–	±	–	–	–	±	±	±
Control		+	+	+	+	+	+	+	+	+

V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

+ : growth positive
- : no growth
± : very little growth

4.7 Molecular characterisation

Molecular characterisation of the isolates was carried out by plasmid profiling and genetic finger printing methods including RAPD and RFLP.

4.7.1 Plasmid profile

Electrophoresis of plasmid preparations of all the isolates on 1per cent agarose gel showed a single DNA band having a relative molecular weight slightly less than 21 kb (Plate 5a). None of the isolates possessed any mega plasmid.

4.7.2 Genetic fingerprinting

Genomic DNA of each of nine isolates was extracted, purified and subjected them to RAPD and RFLP.

4.7.2.1 Isolation of genomic DNA

Genomic DNA was isolated as per the procedure given under 3.10.2.1. Upon electrophoresis on 1 per cent agarose gel, intact DNA was observed in all the isolates accompanied by low molecular weight RNA.

RNase treatment was given for all DNA preparations in order to remove RNA (Plate 5b). The quantity and quality of isolated DNA were checked using spectrophotometer and the data are presented in Table 4.17. Absorbance ratio of the isolates ranged from 1.7 to 1.9 and the yield of DNA, from 2.5 to 7.95μg/ml.

4.7.2.2 Random Amplified Polymorphic DNA (RAPD)

4.7.2.2.1 Screening of primers

Among 15 primers tried, ten primers which gave good amplification with A3, were selected and used for further characterisation of isolates (Plate 5c). The primers selected along with their target sequence are given in Table 4.18.

Table 4.17. Quality and quantity of DNA as revealed by spectrophotometry

Isolate	Optical density		OD_{260}/OD_{280}	Quality of DNA	Quantity of DNA (µg/ml)
	260 nm	280 nm			
V1	0.134	0.074	1.81	Good	3.70
V2	0.085	0.050	1.70	Fairly good	2.50
V3	0.150	0.081	1.85	Good	7.50
K1	0.159	0.088	1.80	Good	7.95
K2	0.133	0.074	1.79	Good	6.65
K3	0.052	0.028	1.85	Good	2.60
A1	0.100	0.056	1.78	Good	5.00
A2	0.080	0.042	1.90	Fairly good	4.00
A3	0.064	0.036	1.77	Good	3.2

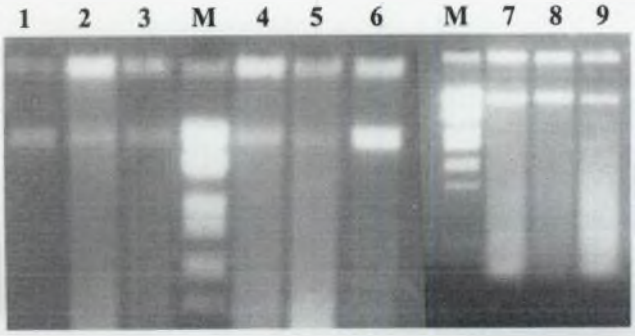
V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

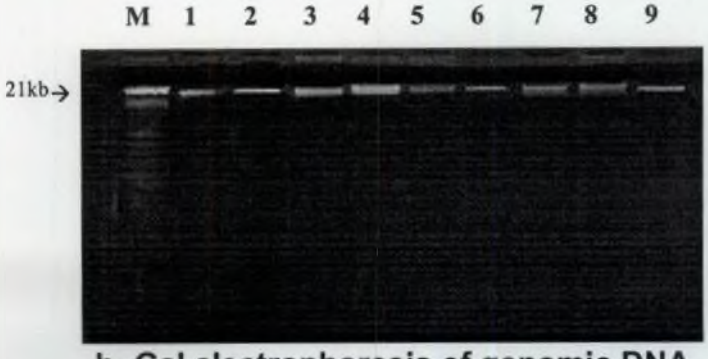
Table 4.18. Details of primers selected for RAPD analysis

Sl. No.	Primer code	Primer sequence
1	OPE 3	CCAGATGCAC
2	OPE 4	GTGACATGCC
3	OPE 12	TTATCGCCCC
4	OPF 5	CCGAATTCCC
5	OPF 6	GGGAATTCGG
6	OPF 7	CCGATATCCC
7	OPF 8	GGGATATCGG
8	OPP 2	TCGGCACGCA
9	OPP 10	TCCCGCCTAC
10	OPAH 8	TTCCGTGCC

Plate - 5

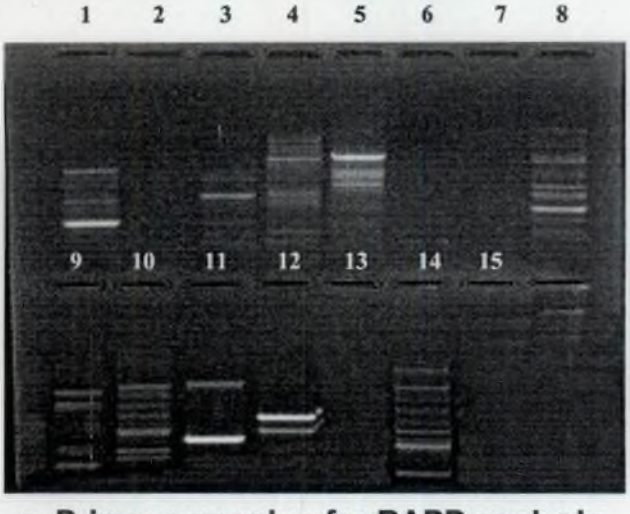


a- Plasmid profile of *R. solanacearum* isolates



b- Gel electrophoresis of genomic DNA

M- Molecular weight marker → λ DNA / *Eco* RI + *Hind* III
Lanes 1 - V1, 2- V2, 3- V3, 4- K1,
5- K2, 6- K3, 7- A1, 8- A2, 9- A3



c- Primer screening for RAPD analysis.

Lanes 1- OPE 3, 2- OPE 5, 3- OPE 4, 4- OPE 12,
5- OPP 2, 6- OPP 3, 7- OPP 5, 8- OPP 10,
9- OPF 5, 10- OPF 6, 11- OPF 7, 12- OPF 8,
13- OPAH 7, 14- OPAH 8, 15- OPAH 9

4.7.2.2.2 RAPD profiles of *R. solanacearum* isolates with selected primers

The total number of bands obtained along with polymorphic bands were recorded to characterise the isolates and the data are given in Table 4.19.

RAPD with OPE 3 showed variation among all nine isolates. Total number of bands ranged from 1 to 9 among the different isolates (Plate 6a).

RAPD using OPE 4 produced wide variation among the isolates and the number of bands ranged from 1 to 10. K1 and K3 shared two common bands. A1, A2 and A3 were also found to share two bands. Of these three Ambalavayal isolates, A2 and A3 were least polymorphic with most of the bands in common (Plate 6b).

Primer OPE 12 showed some similarity among the three Ambalavayal isolates after RAPD sharing two bands in common. Amplification was very weak in all other isolates except K1 (Plate 6c).

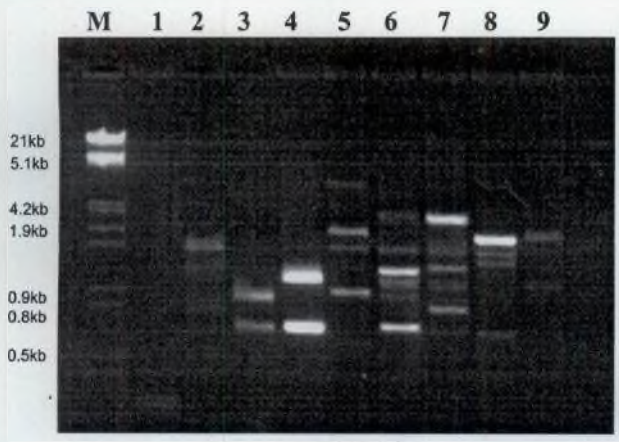
K_1 and K_3 shared one common conspicuous band when OPF 5 was used in RAPD. Amplification was nil in V1 and A2 (Plate 6d).

Among the different isolates, the three Ambalavayal isolates were similar with two common bands on RAPD with OPF 6 (Plate 6e). Only K1 and K3 among Kumarakom isolates showed one common conspicuous band.

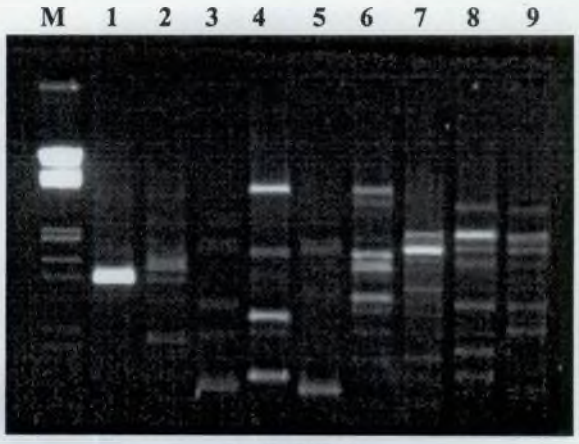
DNA amplification with OPF 7 produced least variation among the isolates. All isolates except V1 shared one common band. No bands were seen in V1. A2 and A3 were more similar sharing two more bands in common. A1 had one polymorphic conspicous band (Plate 7a).

Ambalavayal isolates were similar on RAPD with primer OPF 8 sharing one unique band of approximately 1.45 kb size (Plate 7b). All the other six isolates were highly polymorphic with bands ranging from 2 to 5.

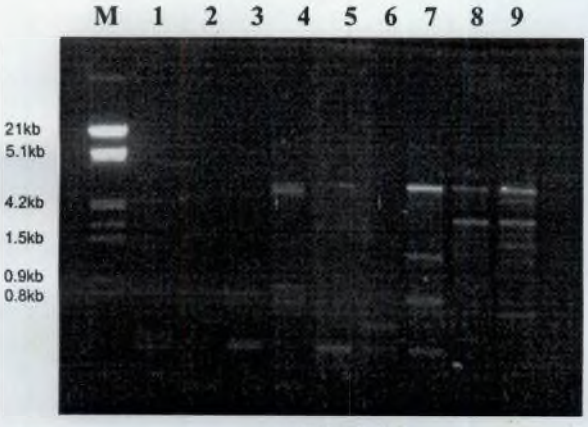
Plate -6
RAPD profiles of *R. solanacearum* with different primers



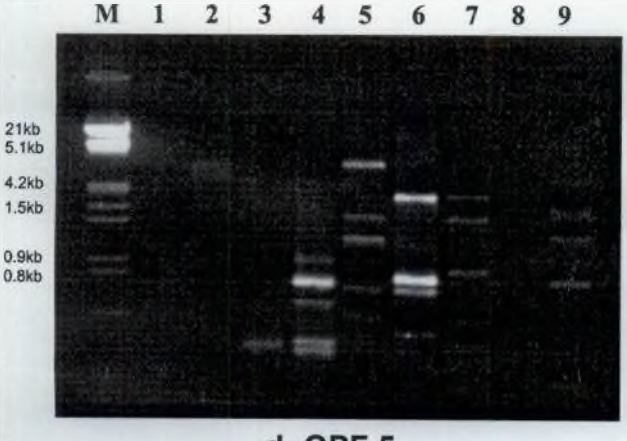
a- OPE 3



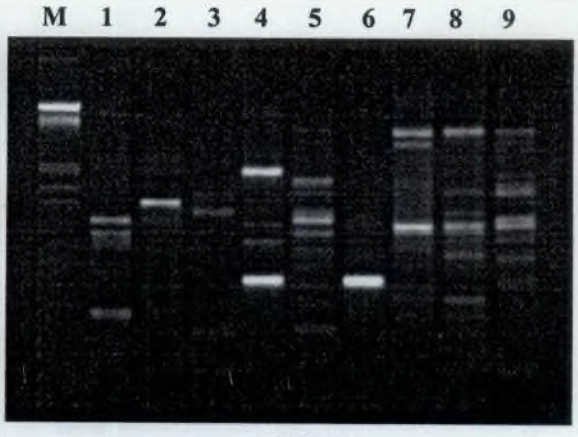
b- OPE 4



c- OPE 12



d- OPF 5

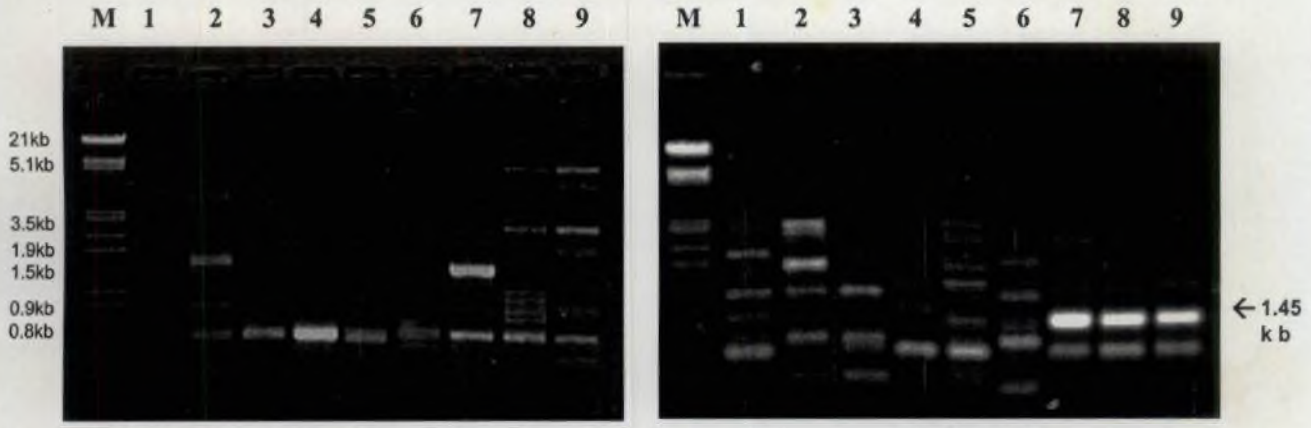


e- OPF 6

M- Molecular weight marker → λDNA / *Eco* RI + *Hind* III

Lanes 1 - V1, 2- V2, 3- V3, 4- K1,
5- K2, 6- K3, 7- A1, 8- A2, 9- A3

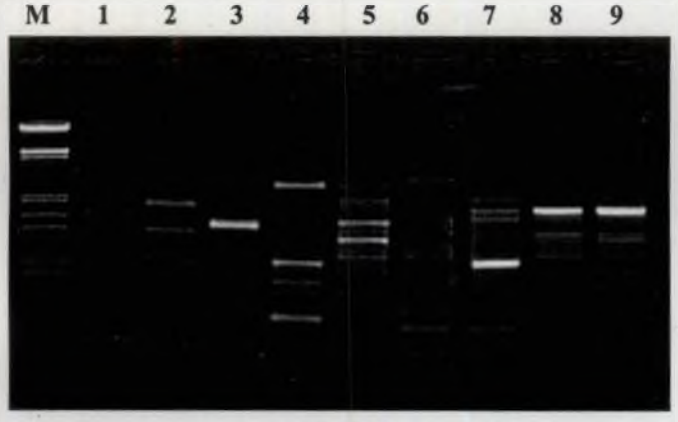
Plate -7
RAPD profiles of *R. solanacearum* with different primers



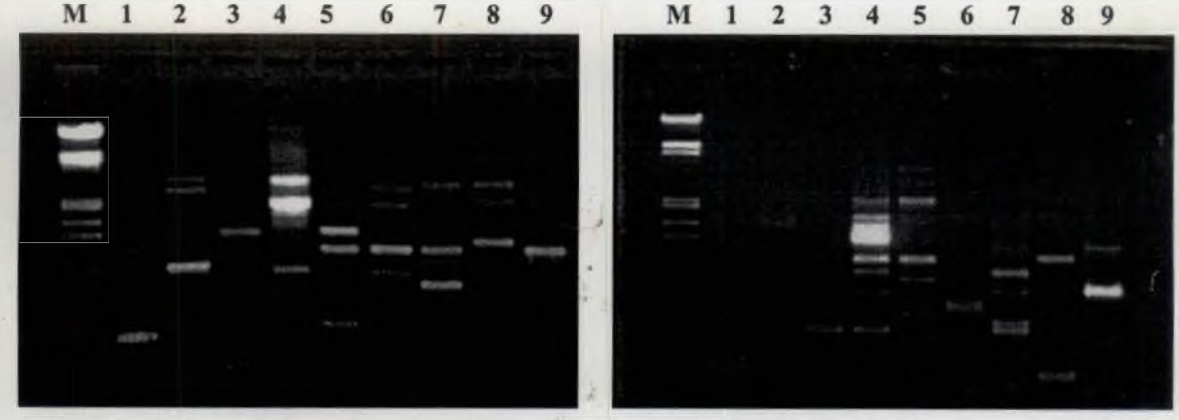
a-OPF 7

b-OPF 8

← indicates unique band common to race 3



c- OPP 2



d- OPP 10

e- OPAH 8

M- Molecular weight marker → λ DNA / *Eco* RI + *Hind* III

Lanes 1 - V1, 2- V2, 3- V3, 4- K1,
5- K2, 6- K3, 7- A1, 8- A2, 9- A3

Table 4.19. The total number of bands along with polymorphic bands obtained with ten primers in RAPD

Isolate	OPE 3		OPE 4		OPE 12		OPF 5		OPF 6		OPF 7		OPF 8		OPP 2		OPP 10		OPAH 8	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
V1	6	0	3	0	2	1	0	0	4	1	0	0	3	0	2	0	2	0	1	0
V2	7	0	4	0	1	0	3	1	3	1	3	1	5	1	4	0	3	0	1	0
V3	6	0	4	0	2	0	1	0	2	0	1	0	3	0	1	0	1	0	1	0
K1	6	1	5	2	5	0	5	4	5	1	1	0	2	0	4	3	5	2	7	1
K2	1	0	7	0	2	0	5	2	7	1	1	0	4	0	7	1	3	1	7	3
K3	5	1	7	0	3	1	4	1	2	0	1	0	3	0	4	1	4	0	1	1
A1	5	1	7	0	6	0	5	2	5	1	2	1	3	0	5	1	5	1	4	1
A2	2	0	3	1	6	1	0	0	6	2	7	3	3	0	5	0	3	1	2	0
A3	3	0	10	0	8	3	5	1	9	0	5	0	3	0	5	0	1	0	2	1

a - Total number of bands b – Number of polymorphic bands

V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

RAPD with primer OPP 2 produced some similarity within A2 and A3 with one conspicous band in common. All other isolates were much dissimilar with number of bands ranging from 1 to 7 (Plate 7c).

K2, K3, A1 and A3 shared one common band on RAPD with OPP 10. A highly amplified band was noticed in K1 (Plate 7d).

RAPD with OPAH 8 showed two bands in common for K1 and K2, one band being common for A2 also. The number of bands amplified among the isolates varied from 1 to 7 (Plate 7e).

The size of the products amplified from *R. solanacearum* isolates with different primers ranged from 0.1 to 5.0 kb.

4.4.2.2.3 Analysis of RAPD profiles

4.4.2.2.3.1 Genetic similarity

Genetic similarity was computed from RAPD profiles as DICE coefficient using the similarity routine of NTSYS PC 2.0 software. The dendrograms were constructed for individual primers and also for the pooled data using UPGMA routine of the above mentioned software.

4.4.2.2.3.2 Individual primers

The genetic similarity ranged from a minimum of zero to a maximum of one for different primers. Primer OPF 8 revealed highest genetic similarity (1) between the isolates, A1, A2and A3 whereas primer OPF 5 showed minimum genetic similarity (0) between all the isolates except V1 and A2. Genetic similarity as DICE coefficients and dendrogram based on the RAPD profiles generated by the individual primers is given in Appendix II.

4.4.2.2.3.3 Pooled data

The genetic similarity matrix pertaining to the pooled data obtained from the ten primers is given in Table 4.20. Highest genetic similarity of 0.56 was

Table 4.20

Genetic similarity as DICE coefficients based on pooled data of RAPD profiles

	V1	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.12	1.00							
V3	0.12	0.25	1.00						
K1	0.18	0.23	0.24	1.00					
K2	0.27	0.27	0.30	0.23	1.00				
K3	0.21	0.27	0.27	0.31	0.28	1.00			
A1	0.19	0.17	0.24	0.34	0.30	0.40	1.00		
A2	0.21	0.25	0.18	0.21	0.32	0.12	0.43	1.00	
A3	0.17	0.26	0.16	0.25	0.34	0.21	0.38	0.56	1.00

Fig. 3

Dendrogram obtained for pooled data of RAPD profiles

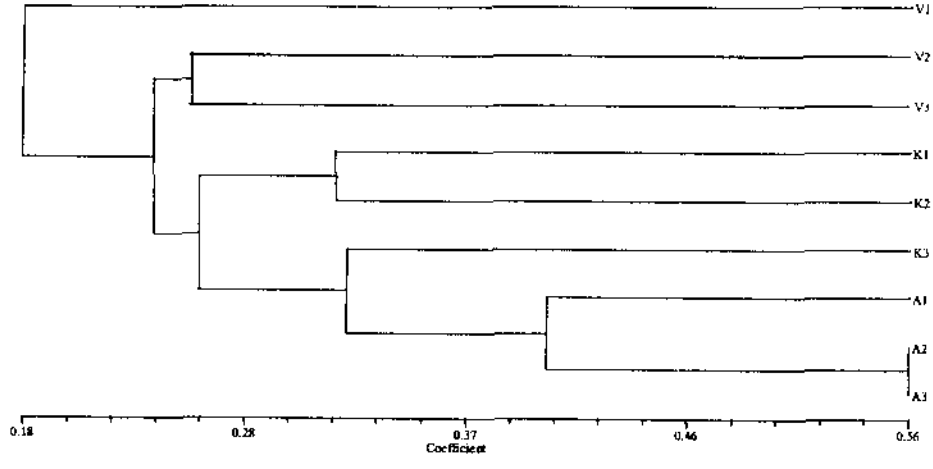


Table 4.21. Summary statistics of RAPD analysis for *R. solanacearum* isolates

Sl.No.	Primer	Total number of bands	Number of polymorphic bands	Per cent polymorphism
1	OPE 3	42	8	19.04
2	OPE 4	63	19	30.15
3	OPE 12	39	10	25.64
4	OPF 5	30	11	36.67
5	OPF 6	66	12	18.18
6	OPF 7	24	9	37.50
7	OPF 8	37	6	16.22
8	OPP 2	39	8	20.51
9	OPP 10	30	5	16.67
10	OPAH 8	29	7	24.14
	Pooled	399	95	23.81

observed between the isolatesA2 and A3, followed by 0.43 between A1 and A2. The dendrogram constructed from the pooled data had five clusters (Fig. 3). The first cluster comprised of A1, A2 and A3 from Ambalavayal. Within the first cluster, A2 and A3 had maximum genetic similarity of 0.56. The second cluster consisted of K2 alone which is separated from the first cluster at genetic similarity value of 0.33. K1 and K2 formed the third cluster having the genetic similarity value of 0.32. The second and third clusters are seen linked at genetic similarity value of 0.26.The fourth cluster is comprised of V2 and V3, and the fifth cluster consisted of V1 alone. The fourth cluster having a genetic similarity coefficient of 0.22 is linked to second and third clusters at a corresponding value of 0.24. The fifth cluster containing V1 alone had a minimum similarity coefficient of only 0.18 showing it highly different from other isolates.

Per cent polymorphism computed for each primer ranged from 16.22 to 37.50 per cent. Primer OPF 7 showed highest per cent polymorphism of 37.50 per cent. The least per cent polymorphism was shown by OPF 8. The summary statistics of RAPD analysis for *R. solanacearum* isolates is given in Table 4.21.

4.7.2.3 RFLP

Genomic DNA digested with four restriction enzymes viz. *Hind* III, *Eco* RI, *Bam* HI and *Pst* I resulted in smear in all isolates. Digestion pattern was found different in the isolates with four different enzymes. Digestion pattern of nine isolates of *R. solanacearum* is given in Table 4.22.

(i) Digestion with *Hind* III

All the nine isolates were digested completely. Smearing was found uniform in A1, A2 and A3 (Plate 8a).

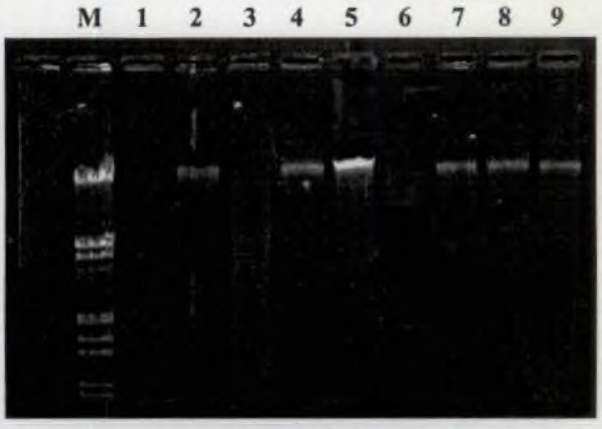
(ii) Digestion with *Eco* RI

V2 and V3 were found only partially digested whereas others were digested completely. In V1, K1 and K3, a very faint smear only was seen compared to others (Plate 8b).

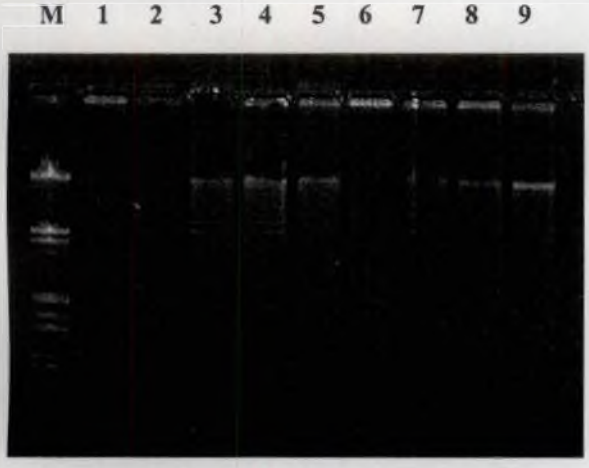
Plate - 8

Restriction analysis of *R. solanacearum* with different restriction enzymes

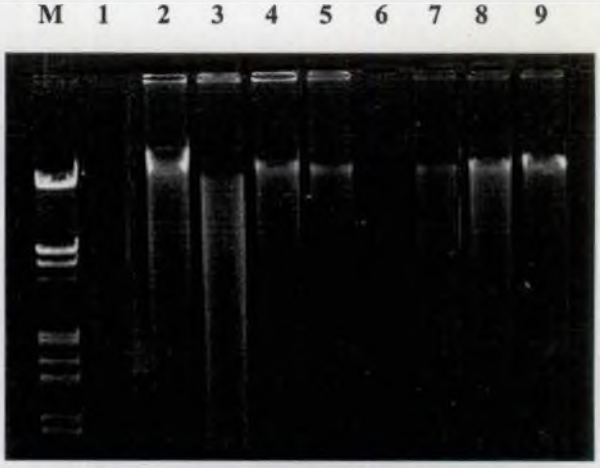
a- *Hind* III



b- *Eco* R I



c- *Bam* H I



d- *Pst* I

M- Molecular weight marker → λ DNA / *Eco* RI + *Hind* III

Lanes 1 - V1, 2- V2, 3- V3, 4- K1,
5- K2, 6- K3, 7- A1, 8- A2, 9- A3

Table 4.22. Digestion pattern of genomic DNA of *R. solanacearum* isolates using restriction enzymes

Isolate	Digestion			
	Hind III	*Eco* RI	*Pst*-I	*Bam* HI
V1	Complete	Complete	Partial	Complete
V2	Complete	Partial	Complete	Complete
V3	Complete	Partial	Complete	Partial
K1	Complete	Complete	Partial	Partial
K2	Complete	Complete	Partial	Partial
K3	Complete	Complete	Complete	Complete
A1	Complete	Complete	Complete	Partial
A2	Complete	Complete	Complete	Partial
A3	Complete	Complete	Partial	Partial

V : Vellanikkara
K : Kumarakom
A : Ambalavayal

1 : Brinjal
2 : Chilli
3 : Tomato

Table 4.23. *R. solancearum* isolates at different temperatures with varying duration

Temperature	Duration	Observation
40°C	48 h	No plasmid curing and profuse growth
41°C	48 h	No plasmid curing and profuse growth
42°C	48 h	No plasmid curing and profuse growth
43°C	12 h	Bacterial cells were killed
44°C	12 h	Bacterial cells were killed
45°C	12 h	Bacterial cells were killed

(iii) Digestion with *Bam* HI

Only V1, V2 and K3 were completely digested whereas others showed only partial digestion (Plate 8c).

(iv) Digestion with *Pst* I

Among nine isolates, V1, K1, K2 and A3 were only partially digested. Complete digestion could be seen in others (Plate 8d).

4.7.2.3.1 Southern hybridization

RAPD with primer OPF 8 yielded a unique band of approximately 1.45 kb size specific to race 3 isolates (Plate 7b). This was used as DNA probe in Southern hybridization. However, no signal was detected on the developed film.

4.8 Role of plasmid in EPS production

Plasmid profiles of mucoid and non-mucoid colonies were compared to assess the role of plasmids in EPS production. Plasmid curing was also tried at high temperatures to ascertain the loss of EPS production traits.

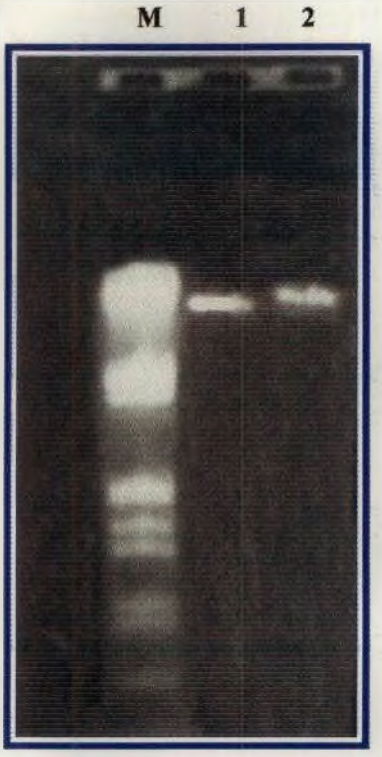
4.8.1 Plasmid profile of mucoid and non-mucoid colonies

Both mucoid and non-mucoid colonies were found to harbour a single plasmid DNA each with slight variation in size (Plate 9a). Plasmid isolated from non-mucoid colony had slightly lower molecular weight compared to mucoid colony.

4.8.2 Plasmid curing

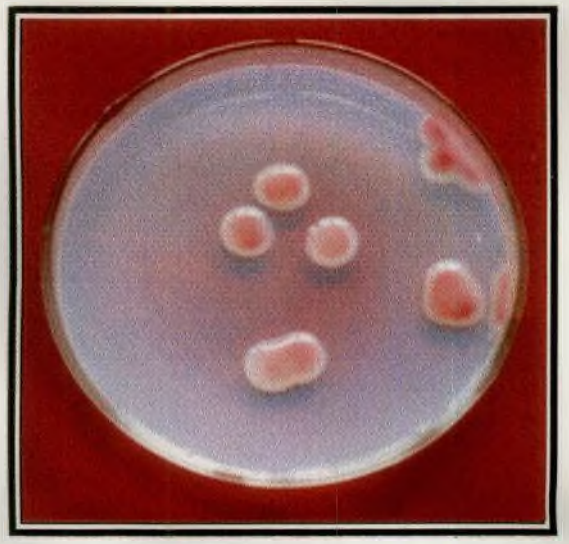
Bacterial isolates were subjected to high temperature ranging from 40°C to 45°C for different intervals of time (Table 4.23). However, all colonies tested for presence of plasmid showed that curing had not taken place. The temperature 43°C and above resulted in the death of bacterial cells whereas temperature below 43°C resulted in mucoid colonies with no variation in colony morphology (Plate 9b).

Plate - 9



a . Plasmid profile of non mucoid and mucoid colonies

M- Molecular weight marker
1 - non mucoid colony
2 -mucoid colony



b . *R. solanacearum* after 48 h of incubation at 42°C on TZC medium

Discussion

5. DISCUSSION

The variability of *Ralstonia solanacearum* has been studied earlier mainly based on cultural, morphological and biochemical characters. In the present study, an attempt has been made to characterise nine isolates of *R. solanacearum* obtained from bacterial wilt affected brinjal, chilli and tomato plants collected from three locations viz., Vellanikkara, Kumarakom and Ambalavayal representing three different agro climatic zones of Kerala, using molecular techniques.

Nine isolates of *R. solanacearum* were isolated on Kelman's triphenyl tetrazolium chloride (TZC) medium to study their cultural and morphological characters. The pathogen formed creamy white colonies with light pink centre on this medium. Several selective media were later developed for the isolation of *R. solanacearum* from soil in which TZC formed the basic component. Since these media were amended with certain antimicrobial compounds to prevent bacterial and fungal contaminants, they were inhibitory to certain strains of *R. solanacearum* (Karganilla and Buddenhagen, 1972; Nesmith and Jenkins, 1979; Granada and Sequeira, 1983; Engelbrecht, 1994). Hence the recovery rate of majority strains of *R. solanacearum* has been reported to be better on TZC medium which could also differentiate wild and variant types of *R. solanacearum*. In the present study, virulent type formed regularly round fluidal white colonies with pink centre, whereas avirulent mutant types formed butyrous, deep red colonies. The relation between pathogenicity and colony type had earlier been reported by Okabe (1949).

All the pure cultures were stored at room temperature as stock suspensions by placing five loopfuls of typical colonies in screw capped tubes containing sterile distilled water. Kelman and Person (1961) had reported that cultures stored in this manner could be maintained relatively free of avirulent mutant types for about 18 to 24 months. However, in the present study, mutant types with non-mucoid colonies could be observed when pure cultures maintained in sterile water were

streaked on TZC medium after 5 to 6 months. Hence, all the isolates had to be subcultured once in three months, in order to maintain their virulence.

In three solanaceous vegetables, viz., brinjal, chilli and tomato, inoculation techniques were standardised for comparing virulence and aggressiveness of the isolates. In brinjal, root dipping was found as the best method of inoculation compared to other methods like stem puncturing, leaf clipping, soil drenching with and without wounding. Inoculation by root dipping in brinjal has been tried earlier by Swanepoel and Young (1988) as well as Paul (1998), though stem puncturing had been used by several workers for testing pathogenicity in brinjal (He *et al.*, 1983; Prior and Steva, 1990). In the present study, based on incubation period and per cent wilt incidence, root dipping was found the most effective technique of inoculation. Rapid disease progress was also noticed in this method of inoculation. In chilli, though the minimum incubation period was found the same in all methods except soil drenching, inoculation by leaf clipping resulted in 100 per cent wilt incidence compared to stem puncturing, soil drenching, soil drenching with wounding and root dipping which were used earlier by Winstead and Kelman (1952). In tomato, leaf clipping and stem puncturing recorded 100 per cent wilt incidence one week after inoculation. But the incubation period was found minimum of five days for leaf clipping, hence it was considered as the best method of inoculation. Wang (1971) suggested leaf clipping as the most reliable and efficient method of inoculation in tomato since the symptoms developed faster and there were less variations among replications. AVRDC (1997) also used leaf clipping for testing efficiency of inoculation in resistant varieties of tomato. Stem puncturing method, which had been widely used by several workers (Kelman, 1954; Husain and Kelman, 1958), also produced high percentage of wilting in the present experiment. Perera *et al.* (1992) reported stem puncturing as the best method of inoculation in bell pepper on comparison with soil drenching with and without root severing with regard to symptom appearance and per cent wilt incidence. However, findings of the present study as root dipping and leaf clipping as the efficient methods of inoculation can be exploited for the mass screening of varieties in breeding programme since both methods are faster and easy to apply as

compared to stem puncuring method. In stem puncturing, there is more chance for damage of plants due to the breaking of leaf axils whereas in leaf clipping, there is less risk of breaking of leaflets from the axils.

Virulence and aggressiveness, used as a measure of pathogenicity of *R. solanacearum* varied considerably for nine isolates on respective susceptible host plants. The virulence of the isolates, calculated based on bacterial wilt index (Emping *et al.*, 1962) on 10th day after inoculation, ranged from 16.67per cent (A1) to 100 per cent (K2). Kumarakom isolates showed more virulence than others whereas the isolates from Ambalavayal were weakly virulent. There are similar reports on variation in virulence of different isolates of *R. solanacearum* when 50 per cent response in symptom expression was compared for the different doses of inoculum (Bora and Addy, 1982). Variation in virulence of the isolates was also reported by He *et al.* (1983) when the pathogenicity was tested on respective hosts and they found some strains as highly virulent whereas some others, being moderate in virulence. Prior and Steva (1990) observed high severity of wilting of *R. solanacearum* isolates from different hosts when tested on respective host plants except in pepper where the isolate produced no wilting. However, the reports made by Kelman and Person (1961) as well as Swanepoel and Young (1988) were contrary to this result as they found the virulence of the isolates as only high on the respective host plants.

When the virulence of the isolates was compared within a specific host, among brinjal and chilli isolates, Kumarakom isolates were found the most virulent. Among tomato isolates, eventhough both Vellanikkara and Kumarakom isolates produced high virulence, Kumarakom isolate was the most virulent when bacterial wilt index on 5 and 10 days after inoculation were compared. The weakly virulent isolates of brinjal, chilli and tomato belonged to Ambalavayal. When the isolates were compared for virulence location wise, brinjal among Kumarakom isolates had moderate severity of wilting on 5 and 10 days after inoculation whereas severity of wilting was high for chilli and tomato isolates. On 15th day after inoculation, brinjal isolate also ranked high in severity of wilting. Among Vellanikkara isolates, brinjal isolate had only moderate severity of wilting even on

15 days after inoculation while other two isolates produced high wilting. All the three isolates of Ambalavayal produced very low severity of wilting even on 15 days after inoculation. Irrespective of the isolates, tomato plants wilted fast after inoculation compared to brinjal and chilli. It could be attributed to the tender tissues of tomato compared to the hardy tissues of brinjal and chilli.

It is a generally accepted fact that virulence could be correlated with the environmental conditions. Under high temperature tropical conditions, wilting will be faster compared to cool humid temperature conditions. In the present study, minimum virulence was recorded by the isolates from Ambalavayal where a cool climate prevails.

Aggressiveness, measured based on incubation period (IP) and latent period (LP_{50}) according to Adhikari (1993) was found highly varying among the nine isolates. Among Vellanikkara isolates, tomato isolate was more aggressive than brinjal and chilli isolates since rapid wilt could be observed only in plants inoculated with tomato isolate. Kumarakom isolates were all highly aggressive compared to isolates of Ambalavayal, where wilting was very slow with high values of incubation period and latent period. Similar results of variation in aggressiveness among *R. solanacearum* strains from tomato plants in Brazil had been reported by Silveira *et al.* (1998). Darasse *et al.* (1997) had ranked different strains of *R. solanacearum* into five aggressiveness patterns differing in behaviour with respect to susceptibility of tomato genotypes and environmental conditions during experimentation. In the present study also, Ambalavayal isolates collected from the cooler climatic conditions could be grouped together since they were the least aggressive compared to others.

In cross inoculation tests, pathogenicity rating differed highly among the isolates. All the isolates from Vellanikkara and Kumarakom were found cross inoculable with one another. Among Vellanikkara isolates, tomato and chilli isolates were found less virulent on brinjal whereas brinjal and tomato isolates were moderately virulent on chilli. But brinjal and chilli isolates produced high virulence on tomato. The symptoms of disease were evident on three to seven days

after inoculation on different hosts. Among Kumarakom isolates, very high virulence was shown by brinjal and chilli isolates on tomato. Ambalavayal isolates were found either non cross inoculable or with very low severity of wilting. Inoculation of brinjal and chilli isolates on tomato as well as that of chilli and tomato isolates on brinjal were found non cross inoculable. On chilli, brinjal and tomato isolates produced very low bacterial wilt index of 5per cent on 10^{th} day after inoculation. Jyothi (1992) and Paul (1998) have reported on the cross inoculability of *R. solanacearum* isolates on different host plants in Kerala.

He *et al.* (1983) conducted pathogenicity tests of 29 strains of *R. solanacearum* collected from China on different host plants like brinjal, chilli, tomato, potato, tobacco, and peanut and they could find that most of the strains were pathogenic to all the host plants except tobacco while a few were non pathogenic to some host plants including tomato, pepper and peanut. The virulence ratings done by Swanepoel and Young (1988) as well as Prior and Steva (1990) were also found supportive to the above results.

The cultural and morphological characters of the isolates were studied on TZC medium. The size of the colony varied from pinpoint to small and moderate. All isolates produced circular creamy white colonies having light pink or reddish pink centre with entire margin. The isolates of Ambalavayal produced reddish pink centered colonies. Fluidity was found highly varying between the isolates. The variation in cultural characteristics of *R. solanacearum* has been reported by He *et al.* (1983) and Prior and Steva (1990). Usually fluidity of the isolates is correlated with virulence and highly virulent colonies tend to be more fluidal which had been reported earlier by Paul (1998) and Mathew *et al.* (2000). In the present study, though the isolates collected from Ambalavayal were highly fluidal, they exhibited a low virulence. This could be due to the difference in climatic conditions prevailing at Ambalavayal, which is comparatively cooler than Vellanikkara and Kumarakom. An increase in the temperature could have led to lower virulence noticed in Ambalavayal isolates inoculated on plants growing at Vellanikkara. In Ambalavayal, high incidence of bacterial wilt is noticed in ginger. In the present study, virulence of the isolates on ginger was not tested. Host preferences of

isolates of *R. solanacearum* cannot be ruled out. Hence more detailed experiments need to be conducted before drawing conclusion regarding virulence of Ambalavayal isolates.

All the nine strains were basically similar in terms of biochemical characteristics. The isolates formed viscous thread like structure with KOH, indicating Gram negative reaction. Testing the solubility of bacterial cells in 3 per cent KOH is an effective supplement to Gram staining, proven to be useful for rapid and accurate differentiation of bacteria originally isolated from plant tissue (Suslow *et al.*, 1982). It is based on the rapid lysis of cell wall of Gram negative bacteria in alkaline solution, releasing DNA which makes the suspension viscous.

All the isolates were found to possess oxidase and catalase enzymes, though there was variation in the extent of enzyme activity. An oxidase-positive reaction is indicative of the presence of cytochrome-c-oxidase in the respiratory electron transport chain (Kovacs, 1956). Among pseudomonads, this test is of important differential value because it forms a confirmatory test for *R. solanacearum*. All Kumarakom isolates and tomato isolate from Ambalavayal recorded delayed positive reaction with oxidase. Catalase functions in converting highly toxic hydrogen peroxide, a byproduct of aerobic respiratory metabolism, to water and oxygen. In the present study, the extent of catalase activity, in the form of quantity of gas bubble production as well as the time taken for it, varied among the isolates. The isolates were negative in arginine hydrolase reaction, indicating their inability to produce ammonia from arginine under anaerobic conditions (Thornley, 1960). Lipase activity was seen positive for all the isolates, which produced white dense precipitate around the isolates on Tween 80 agar developed by Sierra (1957).

Production of fluorescent pigment on King's B agar is a distinguishing feature of fluorescent pseudomonads. In the present study, none of the isolates of *R. solanacearum* produced any fluorescent pigment. However, a nonfluorescent brown diffusing melanin like pigment was formed by the isolates in a medium

containing one per cent tyrosine. Variation in levan production from glucose was observed among the isolates. Vellanikkara and Kumarakom isolates did not produce any levan. However, Ambalavayal isolates produced slightly raised colonies, indicating levan production on the medium supplemented with 5 per cent sucrose. Earlier reports indicate that isolates of *R. solanacearum* do not produce any levan (Hayward, 1964).

In glucose oxidation test, glucose was seen oxidised in both sealed and unsealed tubes in all isolates. Eventhough, *R. solanacearum* is oxidative, glucose oxidation was found to take place in sealed tubes also. Colour change of medium from green to yellow in sealed tubes could be attributed to entry of oxygen through the space developed under agar seal during gas formation (Plate 3a). In denitrification test, the isolates of Vellanikkara utilized nitrate as an alternate terminal electron acceptor in the absence of oxygen, thus resulting in the evolution of nitrogen gas and oxides of nitrogen. Thus, disruption of agar seal was evident within 24 to 48 h in Vellanikkara isolates (Plate 3b&3c). However, Kumarakom and Ambalavayal isolates did not utilize nitrate, hence no disruption of agar seal was seen. Similar variation in biochemical tests had been reported earlier by He *et al.* (1983) and Prior and Steva (1990).

On the basis of utilization of carbohydrates, the isolates were differentiated into biovars III and IIIA. Among the nine isolates, brinjal and chilli isolates of Vellanikkara, brinjal and tomato isolates of both Kumarakom and Ambalavayal, which utilised all the carbohydrates were grouped into biovar III. Tomato isolate of Vellanikkara and chilli isolates of Kumarakom and Ambalavayal, differed from the others in their ability to oxidize dulcitol alone and were designated as biovar IIIA. In all the nine isolates, differences were observed with respect to time of utilization of carbohydrates. However, these differences were not taken into consideration for classifying the isolates into biovars. There are reports on the occurrence of biovars III, IV and V on different host plants in Kerala (Devi, 1978; Paul, 1998; Jyothi, 1992 and Mathew *et al.*, 2000). Biovar IIIA was first reported in Himachal Pradesh on brinjal, tomato and capsicum (Kumar *et al.*, 1993). Hence this is the first report of biovar IIIA in Kerala. Biovar III has been reported from solanaceous

hosts as early as 1964 by Hayward. Tabei and Quimio (1978) as well as Valdez (1985), based on their studies conducted in Philippines, had confirmed that in solanaceous crops, biovar III was predominant. Contrary to the report of biovar IV (Devi, 1978) and biovar V (Paul, 1998) in brinjal, in the present study, all isolates from brinjal were found to belong to biovar III. Virulence of the isolates could not be correlated with different biovars. This is in agreement to reports of virulence having no relation with inoculum potential (Buddenhagen, 1985 and Morgado *et al.*, 1992). On the other hand, some studies (Martins *et al.*, 1998; Prior *et al.*, 1994 and Lopes *et al.*, 1994) have found positive correlation between biovar and virulence.

Race differentiation of the isolates was done based on the hypersensitivity reaction of the isolates on capsicum leaves according to Lozano and Sequeira (1970). The isolates of Vellanikkara and Kumarakom produced either water soaking lesions on first day followed by necrotic lesion with yellow halo on third day or wilting within ten days and were grouped under race 1. The isolates from Ambalavayal produced only yellowish discolouration of the infiltrated area and were grouped as race 3. Buddenhagen *et al.* (1962) had separated several hundred isolates of *R. solanacearum* into three races, differing mainly in host ranges. According to him, tobacco, tomato and many solanaceous crops were affected by race 1. Though race 3 affected potatoes and tomatoes, it was only weakly virulent on other solanaceous crops. This is supportive to the present results since the six isolates obtained from brinjal, chilli and tomato from Vellanikkara and Kumarakom belonged to race 1. The other three isolates obtained from Ambalavayal classified under race 3, were moderately virulent. The hypersensitivity reaction induced by *R. solanacearum*, however, is dependent upon the population in the inoculum, the period of exposure to light, and the temperature incubation, since these factors evidently influence the type of reaction obtained (Lozano and Sequeira, 1970). The variation in these factors can change the type of symptoms produced. Hence, in the present study, wilting of plants without any necrotic lesion could be attributed to any one of these factors. The existence of race 1 has already been reported earlier, in Kerala (Paul, 1998 and Mathew *et al.*,

2000). Prior and Steva (1990) conducted studies on 24 strains of *R. solanacearum* collected from Martinique and Guadeloupe in French West Indies. Among these, one strain isolated from the cooler area of Guadelope having low temperature and higher elevation was identified as race 3. In the present study also, since race 3 was identified only among the isolates of Ambalavayal, it could be attributed to the cooler climate prevailing there. Tsuchiya and Horita (1997) correlated biovars and races, and they divided race 1 strains of Japan into four biovars, of which biovar IV was most common. However, in the present study, no such correlation among biovars and races could be noticed. Both biovars III and IIIA could be detected among race 1 as well as race 3. However, biovar III was predominant in both.

The intrinsic antibiotic resistance pattern varied among the different isolates of *R. solanacearum.* Though all the nine isolates were resistant to ampicillin and sensitive to chloramphenicol, they differed from one another in their response to other antibiotics tried. Prior and Steva (1990) and He *et al.* (1983) reported that several strains of *R. solanacearum* were susceptible to ampicillin, but resistant to chloramphenicol. Prior and Steva (1990) commented that the difference in antibiotic sensitivity could be due to evolution or introduction of new strains of *R. solanacearum* since they could notice variation in resistance pattern of 24 strains to the same antibiotic. In the present study also, resistance pattern varied greatly among isolates. This variation is quite natural because drug resistance genes are usually located on plasmids, which are dispensable.

In order to characterise the isolates at molecular level, the plasmid profile of the nine isolates was developed. The plasmid DNA was first isolated using alkali lysis method developed by Birnboim and Doly (1979). Later a much better protocol was used for the isolation of plasmid DNA which yielded good quality DNA in much lesser time (Santha, 2001). All isolates harboured a single plasmid DNA which migrated slightly faster than 21 kb fragment of λDNA *Hind* III *Eco*RI double digest. Earlier reports also indicate presence of one or two plasmids in isolates of *R. solanacearum.* Morales and Sequeira (1985) reported a single plasmid in 14 of the 22 isolates investigated. In the present study, isolation of megaplasmid was also tried using the method given by Boominathan and

Mahadevan (1988). However, no megaplasmid was obtained. Rosenberg *et al.* (1982) reported that megaplasmid bands present in *R. solanacearum* were less easily detected. Temperature treatment of DNA sample between 50 and 95°C for 20 to 30 minutes can denature the chromosomal DNA without affecting plasmid DNA bands (Kado and Liu, 1981). Hence it may be possible to isolate megaplasmid from bacteria by treating the samples at high temperature.

Genomic DNA was extracted from all the isolates to carryout RAPD and RFLP for genetic fingerprinting. A single discrete band of genomic DNA of good quality was obtained in all isolates. Due to the interference of excess of polysaccharides, it was very difficult to get pure DNA. Even 0.1per cent NaCl wash given twice could not eliminate the problem. Since the purity of DNA was found to inhibit DNA amplification in RAPD, isolated DNA samples were given RNase treatment followed by phenol: chloroform: isoamyl alcohol extraction to get pure and intact DNA. The quantity of DNA samples ranged from 2.50 to 7.95 µg/ml and their quality was confirmed by spectrophotometer reading.

Genetic fingerprinting using RAPD revealed much diversity among the isolates. Out of a total of 15 primers screened, ten primers that gave good amplification were selected for doing RAPD. In RAPD, all the ten primers tested exhibited high polymorphism with respect to different isolates. The number of bands ranged from zero to ten and size of amplified fragments, from 0.1 to 5 kb. DNA amplification with different primers revealed great diversity in the population of *R. solanacearum* biovars III and IIIA. None of the primers yielded bands specific to any of the two biovars. There are reports on the genetic diversity among the biovars of the pathogen (Jaunet and Wang, 1997), which claim that the population of *R. solanacearum* strains cannot be genetically differentiated at biovar level. Hence, molecular typing using RAPD revealed more diversity compared to biovar typing which has also been reported by Darasse *et al.* (1997).

High polymorphism was exhibited among the isolates of race 1 compared to race 3. Certain primers including OPE 3, OPE 12, OPF 6, OPF 7 and OPF 8 produced very less polymorphism among the three race 3 isolates, thus sharing

some monomorphic bands. Among these primers, OPF 8 was found to yield a unique band of approximately 1.45 kb size for race 3 isolates from Ambalavayal. This band was absent in all other isolates (Plate 7b). Hence this could be considered as a molecular marker for identification of race 3 of *R. solanacearum* in Kerala. Though all the three isolates of race 3 share a common band, there are definitely not the same strain since these are not cross inoculable and exhibited different banding pattern with other primers. Van Der Wolf *et al.* (1997) have shown the genetic diversity among *R. solanacearum* race 3 isolates in Western Europe using other molecular techniques like AFLP (Amplified Fragment Length Polymorphism) and Rep-PCR (Repetitive PCR).

Genetic similarity values obtained based on the RAPD profiles showed minimum similarity value of 0.00 computed by DICE coefficient with all the primers except OPF 8 indicating that the primers are efficient in distinguishing all the nine isolates. Hence this explains the polymorphism obtained among the isolates. Primer OPF 8, which produced a unique band specific to Ambalavayal isolates, showed maximum similarity value of 1 among these three isolates. Other primers also showed higher genetic similarity values among them compared to race 1 isolates.

Dendrogram obtained from the pooled data of RAPD profiles showed five clusters. Clustering of the nine isolates with ten primers using UPGMA routine indicated that race 3 isolates formed a different cluster. This confirmed the less diversity among isolates from Ambalavayal. Of these race 3 isolates, chilli and tomato isolates had maximum genetic similarity with DICE coefficient value of 0.56. Among Kumarakom isolates, chilli isolate was seen as a different cluster separated from brinjal and tomato isolates, which formed the third cluster. Chilli and tomato isolates from Vellanikkara formed the fourth cluster whereas brinjal isolate was separated from them forming the fifth cluster. Brinjal isolate from Vellanikkara was found highly diverse among the nine isolates with very low genetic similarity value.

An attempt was made to distinguish the isolates by restriction analysis of genomic DNA using *Hind* III, *Eco*RI, *Bam* HI and *Pst* I. However, restriction yielded only smear without any detectable bands. Hence it could not be used as an evident tool for characterising the isolates eventhough there was some difference in the smearing pattern of the digested DNA in the different isolates. Many earlier reports also indicate only smearing of the genomic DNA (Negishi *et al.*, 1990 and Fegan *et al.*, 1997). In contrary to this, there are other reports on the differential banding pattern obtained in a bacteria, *Pasteurella multocida* after digesting the genomic DNA with *Eco* RI, *Hind* III and *Hha*I (Wilson *et al.*, 1992).

Since mere restriction analysis could not characterise the bacterial isolates, Southern hybridisation was carried out with restricted genomic DNA using a race 3 specific probe obtained after RAPD with OPF 8 (Plate 7b). No hybridisation signal could be obtained with the probe. There are reports on the DNA probe obtained after subtractive hybridisation specific for *R. solanacearum* race 3 (Cook and Sequeira, 1991a). Lee and Wang (2000) have developed a 0.7 kb DNA probe to detect a 2.7 kb *Eco*RI fragment from a *R. solanacearum* strain useful for diagnostic and detection tests.

In order to assess the role of plasmid in exopolysaccharide (EPS) production, heat curing was tried and it turned out unsuccessful. The temperatures 43°C and above for different duration resulted in the death of bacterial cells while the temperatures below 43°C produced mucoid bacterial colonies without any curing. Morales and Sequeira (1985) have reported the failure to find cured derivatives of certain strains after treatment with acridine orange. The role of plasmid in EPS production was assessed also by comparing the plasmid profile of both mucoid and non-mucoid colonies. Both type of colonies yielded a single plasmid DNA band. The non-mucoid avirulent colony harboured a plasmid band which has slightly lower molecular weight than that of mucoid colony (Plate 9a). The reduction in the size of plasmid could be attributed to the deletion and/or addition of some genes on the plasmid (Gadewar *et al.*, 1998). However, single plasmid band detected both in mucoid and non-mucoid forms of *R. solanacearum* indicated no loss of plasmid during phenotype conversion. Morales and Sequeira

(1985) and Gadewar *et al.* (1998) also have reported similar results in isolates forming stable nonmucoid forms and they had observed no change in their size of plasmid DNA. However, a miniplasmid present in avirulent forms has been reported earlier by Negishi *et al.* (1990), supporting our results.

In the present study, though wide diversity was revealed among the nine isolates at molecular levels, a molecular marker specific for race 3 isolates could be detected using RAPD, which can be used to identify race 3 of *R. solanacearum* in Kerala. However, more isolates are to be screened carefully before recommending it as a molecular marker for race 3.

Summary

6. SUMMARY

An investigation on 'Molecular characterisation of *Ralstonia solanacearum* causing bacterial wilt in solanaceous vegetables' was undertaken in the Department of Plant Pathology and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara from December 1999 to August 2001. The objectives of the study were to charaterise the bacterial wilt pathogen, *Ralstonia solanacearum*, collected from brinjal, chilli and tomato from three locations representing three different agro climatic zones of Kerala, at cultural, morphological, biochemical and molecular levels. The salient findings of the study are summarised below.

1. *R. solanacearum* causing wilt in brinjal, chilli and tomato, was isolated from three different locations viz., Vellanikkara, Kumarakom and Ambalavayal, on TZC medium.

2. Single colonies of pure cultures were transferred into sterile water and the bacterial suspensions were maintained at room temperature. The virulence of the isolates was checked once in three months.

3. Inoculation techniques for testing pathogenicity on brinjal, chilli and tomato were standardised. Root dipping was found to be the most effective method of inoculation in brinjal, whereas in chilli and tomato, leaf clipping was the best.

4. Virulence of the isolates was assessed on the respective host plants based on BWI at 5, 10 and 15 days after inoculation. All isolates from Kumarakom showed high BWI indicating high virulence. Vellanikkara isolates were moderately virulent whereas Ambalavayal isolates, weakly virulent.

5. Aggressiveness of the isolates was measured based on incubation period (IP) and latent period (LP_{50}). All isolates from Kumarakom were found highly aggressive with minimum IP and LP_{50}. Brinjal isolate from Vellanikkara, and chilli and tomato isolates from Ambalavayal were found, less aggressive.

6. Cross inoculability of the isolates was found highly varying with each location. All Vellanikkara and Kumarakom isolates were found cross inoculable whereas Ambalavayal isolates were not.

7. Cultural and morphological characters of the isolates were studied on TZC medium. All isolates formed creamy white colonies with pink or reddish pink centre and their size varied from pinpoint to small. The fluidity of the isolates was found highly varying. The isolates were Gram negative, rod shaped and motile.

8. Biochemical characterisation of the isolates was done using nine different tests. All isolates gave positive response with 3 per cent KOH, 3 per cent H_2O_2, oxidase test, glucose oxidation and lipase activity. The isolates were negative for fluorescent pigment and production of ammonia from arginine. Variation in response of the isolates was noticed with levan production and denitrification.

9. Biovar differentiation was carried out based on the capability of isolates to utilise disaccharides and hexose alcohols. Biovars III and IIIA were identified among the nine isolates. Tomato isolate from Vellanikkara and chilli isolates from Kumarakom and Ambalavayal which could not utilize dulcitol were grouped under biovar III A and the other six, which utilized all carbohydrates, under biovar III.

10. Race identification of isolates was done based on hypersensitivity reaction on capsicum leaves. Races 1 and 3 were identified among the isolates. All Vellanikkara and Kumarakom isolates belonged to race 1 whereas the three Ambalavayal isolates were grouped under race 3.

11. Intrinsic antibiotic resistance pattern of the different isolates was studied. All isolates were found resistant to ampicillin and sensitive to chloramphenicol. The isolates showed varying response to the other antibiotics tested.

12. Plasmid profiles of the nine isolates were compared and not much difference could be detected in the plasmid DNA obtained from different isolates. A single plasmid DNA of molecular weight slightly less than 21 kb was present in all the isolates. None of the isolates possessed megaplasmid.

13. Genomic DNA extracted from all the isolates were of good quality and quantity. The quantity of isolated DNA ranged from 2.5 to 7.95 µg/ml.

14. RAPD using ten decamer primers, revealed high polymorphism among the isolates. The number of bands ranged from zero to ten and size of amplified fragments from 0.1 to 5 kb. Great diversity was noticed among biovars III and IIIA as well as among race 1 isolates. Race 3 isolates showed less polymorphism with certain primers. OPF 8 yielded a unique band of approximately 1.45 kb size for race 3 isolates.

15. Dendrogram constructed from the pooled data of ten RAPD profiles showed five clusters in which Ambalavayal isolates formed a separate cluster with high genetic similarity between them.

16. Restriction digestion of the isolates in RFLP with four restriction enzymes, showed only smearing pattern of the DNA, without any bands. In Southern hybridization, no signal could be detected on X-ray film.

17. Plasmid profiles of both mucoid and non-mucoid colonies were compared to assess the role of plasmid in EPS production. Both mucoid and non-mucoid colonies were found to possess a single plasmid though there was slight reduction in size of plasmid in non-mucoid colonies.

18. Heat curing of the isolates was done to knock out plasmid DNA from the bacterial cells. No curing was observed with the isolates at higher temperatures with different duration.

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* Originals not seen

Appendices

APPENDIX I

A. Composition of media

1. Triphenyl tetrazolium chloride (TZC) medium

Peptone	-	10.0 g
Casein hydrolysate	-	1.0 g
Glucose	-	5.0 g
Agar	-	20.0 g
Distilled water	-	1 l
pH	-	7.0

1% TZC was added to a final concentration of 5 ml/ l after autoclaving.

2. *King's B medium*

Peptone	-	2.0 g
Glycerol	-	1.0 g
K_2HPO_4	-	0.15 g
$MgSO_4$	-	0.15 g
Distilled water	-	100 ml
pH	-	7.2-7.4
Agar	-	2.0 g

3. Thornley's semisolid medium

Bacto peptone	-	1.0 g
NaCl	-	5.0 g
K_2HPO_4	-	0.3 g
Phenol red	-	0.01 g
L-Arginine hydrochloride	-	10.0 g
Bacto Agar	-	3.0 g
Distilled water	-	1 l
pH	-	7.2

4. Hayward's semisolid medium

$NH_4H_2PO_4$	-	1.0 g
KCl	-	0.2 g
$MgSO_4.7H_2O$	-	0.2 g
Bacto peptone	-	1.0 g
Bromthymol blue	-	0.08 g
Distilled water	-	1 l
pH adjusted to	-	7.0 – 7.1
Agar	-	3.0 g

5. Van den Mooter Succinate (VMS) medium

KH_2PO_4	-	0.5 g
K_2HPO_4	-	0.5 g
$KgSO_4.7H_2O$	-	0.2 g
Sodium succinate	-	2.0 g
KNO_3	-	3.0 g
Yeast Extract	-	5.0 g
Distilled water	-	1 l
pH	-	6-7
Bacto Agar	-	3.0 g

6. Van den Mooter Glycerol (VMG) medium

KH_2PO_4	-	0.5 g
K_2HPO_4	-	0.5 g
$MgSO_4.7H_2O$	-	0.2 g
Glycerol	-	2.0 g
KNO_3	-	3.0 g
Yeast Extract	-	5.0 g
Distilled water	-	1 l
pH	-	6-7
Bacto Agar	-	3.0 g

2. **Lysis buffer**

200 mM NaOH

1% SDS

3. **Neutralization buffer (for plasmid DNA isolation)**

3 M potassium acetate pH 4.8

4. **TES buffer**

1 M Tris pH 8	-	5 ml
0.5 M EDTA	-	10 ml
Sucrose	-	20.5 g
Water	-	200 ml

5. **TE buffer**

Tris	-	1.211 g
EDTA	-	0.372 g
Water	-	1 l
pH	-	7.5 (with acetic acid)

6. **Gel loading dye**

Glycerol	-	60%
TAE buffer	-	30%
1% Bromophenol blue	-	10%

7. **TAE buffer 50 X**

Tris base	-	242 g
Glacial acetic acid	-	57.1 ml
0.5 M EDTA	-	100 ml
pH	-	8.0

Make up with distilled water to 1 l.

8. Depurination solution

HCl	-	0.2 N

9. Denaturation solution

NaCl	-	1.5 M
NaOH	-	0.5 M

10. Neutralization solution (for Southern blotting)

NaCl	-	1 M
Tris HCl	-	1 M (pH 7.4)

11. 20 X SSC solution

NaCl	-	87.65 g
Sodium citrate	-	44.1 g
Sterile MilliQ water	-	500 ml
pH	-	7.0

12. *Prehybridization solution*

6 X SSC

5 X Denhardt's reagent

100 μg/ml denatured fragmented salmon sperm DNA

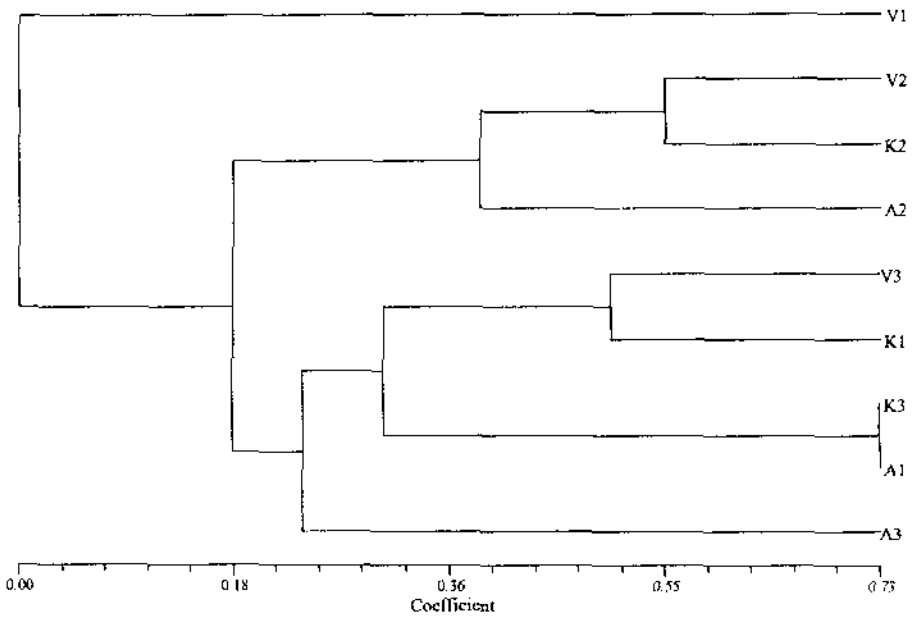
13. Denhardt's reagent (50 X)

Polyvinyl pyrolidone	-	1.0 g
Ficoll (Type 400, Pharmacia0	-	1.0 g
Bovine serum albumin	-	1.0 g
(Fraction V, Sigma)		
Sterile milliQ water	-	100 ml

APPENDIX II

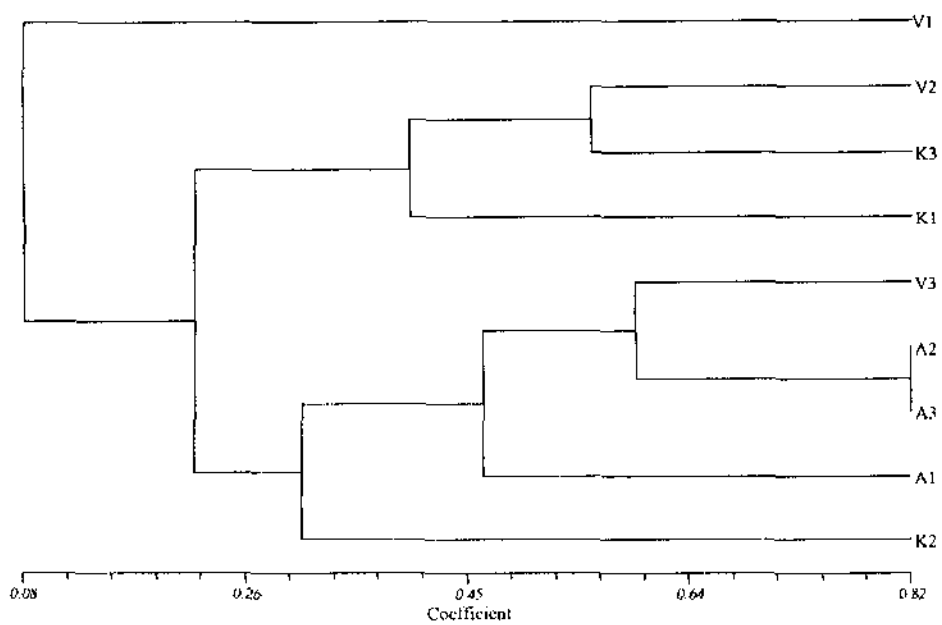
1.Genetic similarity as DICE coefficients and dendrogram based on the RAPD profiles generated by the primer OPE-3

	VI	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.00	1.00							
V3	0.00	0.33	1.00						
K1	0.00	0.33	0.50	1.00					
K2	0.00	0.55	0.00	0.00	1.00				
K3	0.00	0.40	0.31	0.31	0.44	1.00			
A1	0.00	0.27	0.31	0.31	0.22	0.73	1.00		
A2	0.00	0.44	0.00	0.00	0.33	0.13	0.38	1.00	
A3	0.00	0.00	0.00	0.50	0.22	0.31	0.15	0.00	1.00



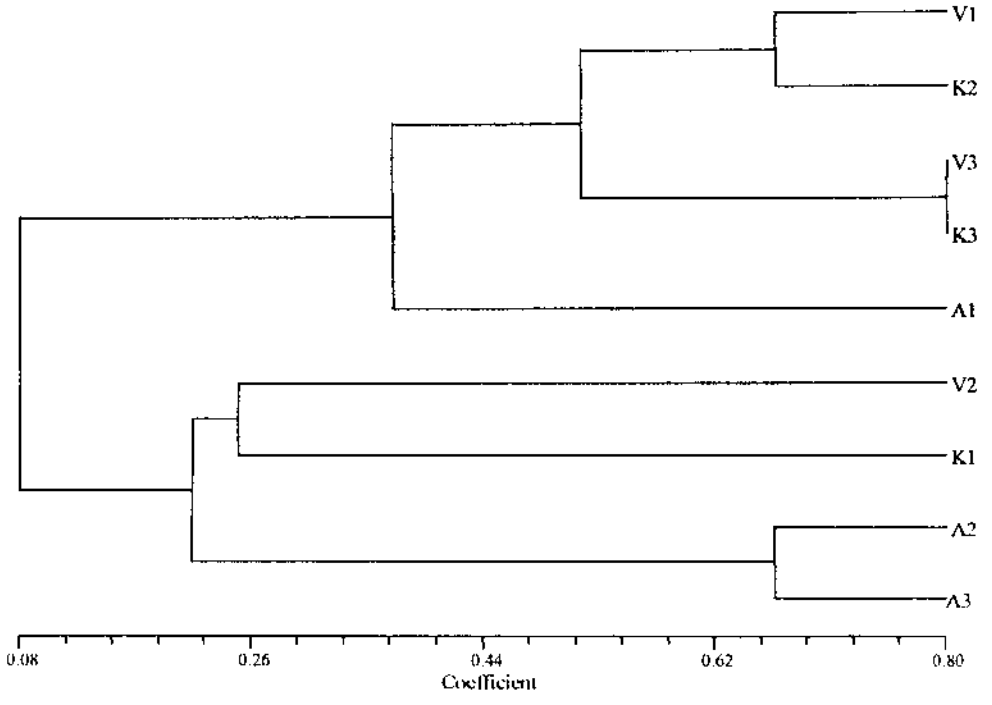
2. Genetic similarity as DICE coefficients and dendrogram based on the RAPD profiles generated by the primer OPE-4

	V1	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.22	1.00							
V3	0.00	0.31	1.00						
K1	0.22	0.25	0.15	1.00					
K2	0.00	0.00	0.29	0.00	1.00				
K3	0.18	0.56	0.27	0.56	0.00	1.00			
A1	0.00	0.25	0.31	0.38	0.20	0.33	1.00		
A2	0.00	0.35	0.57	0.35	0.36	0.21	0.59	1.00	
A3	0.00	0.25	0.62	0.25	0.40	0.22	0.50	0.82	1.00



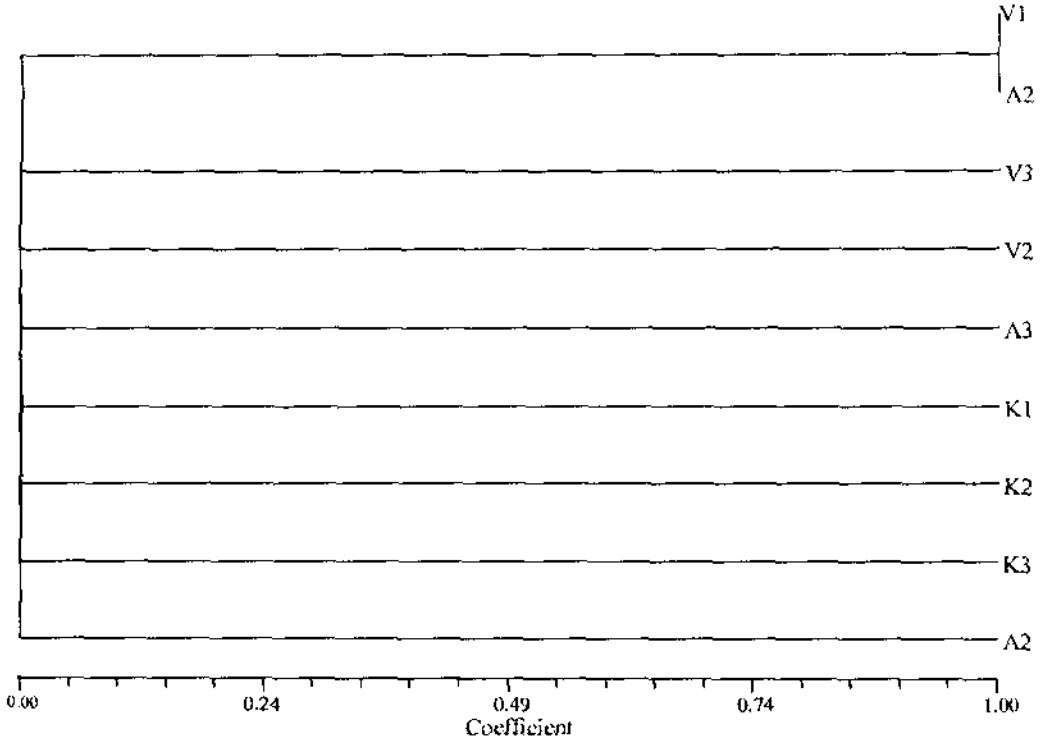
3.Genetic similarity as DICE coefficients and dendrogram based on the RAPD profiles generated by the primer OPE-12

	V1	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.00	1.00							
V3	0.50	0.00	1.00						
K1	0.00	0.25	0.25	1.00					
K2	0.67	0.00	0.67	0.00	1.00				
K3	0.40	0.00	0.80	0.22	0.50	1.00			
A1	0.25	0.00	0.50	0.33	0.29	0.44	1.00		
A2	0.22	0.22	0.00	0.15	0.00	0.00	0.31	1.00	
A3	0.00	0.20	0.00	0.29	0.00	0.00	0.29	0.67	1.00



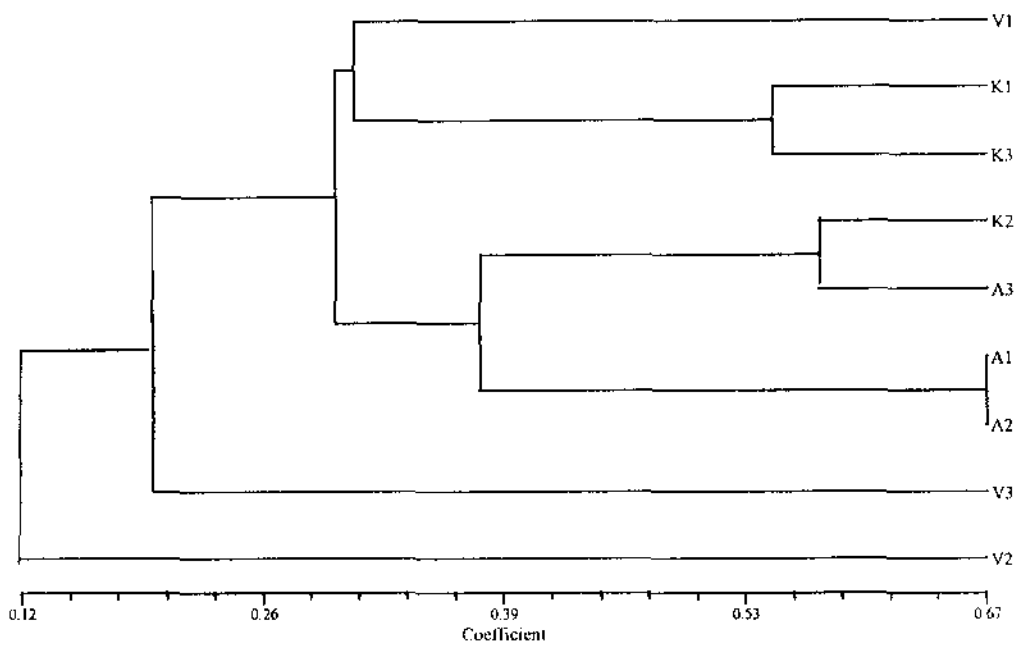
4. Genetic similarity as DICE coefficients and dendrogram based on the RAPD profiles generated by the primer OPF-5

	V1	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.00	1.00							
V3	0.00	0.00	1.00						
K1	0.00	0.22	0.00	1.00					
K2	0.00	0.40	0.00	0.18	1.00				
K3	0.00	0.00	0.00	0.15	0.14	1.00			
A1	0.00	0.00	0.00	0.17	0.15	0.13	1.00		
A2	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	
A3	0.00	0.44	0.00	0.20	0.00	0.31	0.17	0.00	1.00



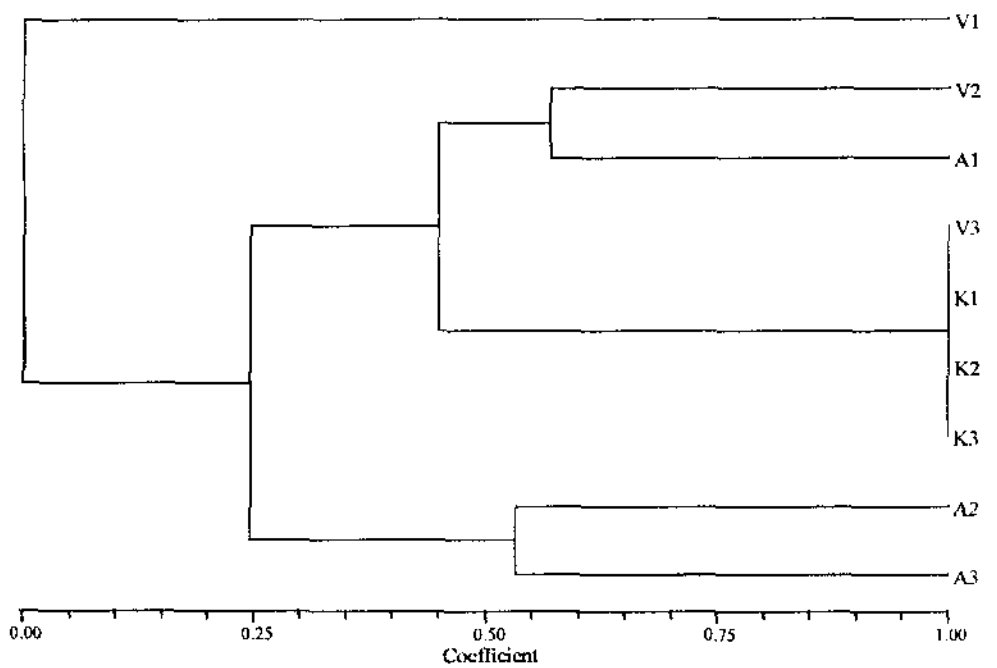
5. Genetic similarity as DICE coefficients and dendrogram based on the RAPD profiles generated by the primer OPF-6

	V1	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.00	1.00							
V3	0.00	0.17	1.00						
K1	0.33	0.13	0.31	1.00					
K2	0.29	0.24	0.27	0.33	1.00				
K3	0.29	0.00	0.25	0.55	0.46	1.00			
A1	0.36	0.00	0.17	0.40	0.35	0.40	1.00		
A2	0.27	0.11	0.25	0.21	0.38	0.14	0.67	1.00	
A3	0.27	0.33	0.13	0.32	0.57	0.14	0.33	0.45	1.00



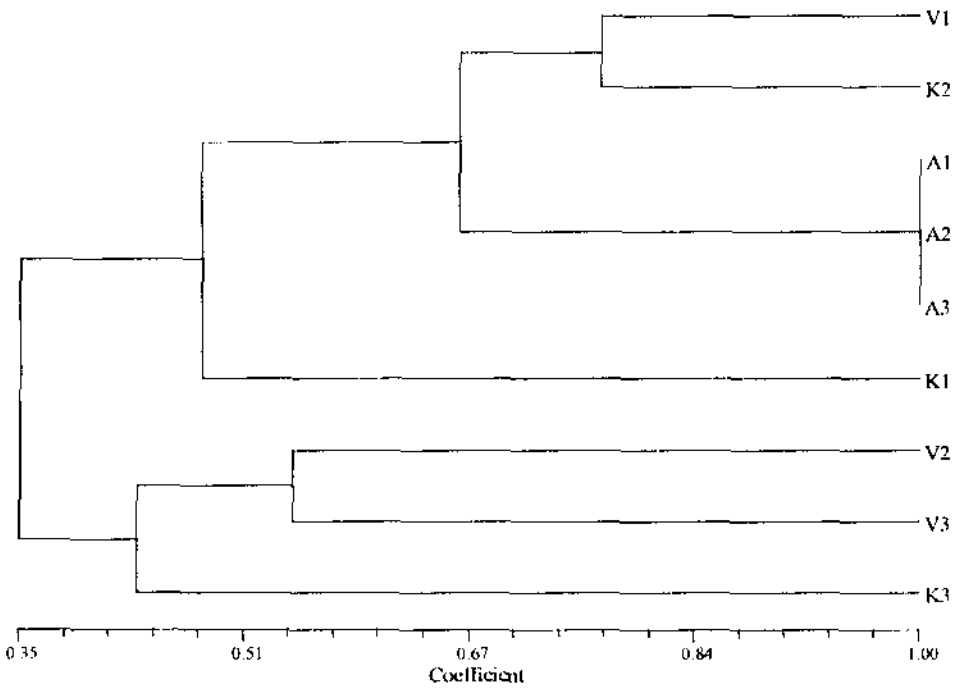
6. *Genetic similarity* as DICE coefficients and dendrogram based on the RAPD profiles generated by the primer OPF-7

	V1	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.00	1.00							
V3	0.00	0.40	1.00						
K1	0.00	0.40	1.00	1.00					
K2	0.00	0.40	1.00	1.00	1.00				
K3	0.00	0.40	1.00	1.00	1.00	1.00			
A1	0.00	0.57	0.50	0.50	0.50	0.50	1.00		
A2	0.00	0.33	0.22	0.22	0.22	0.22	0.18	1.00	
A3	0.00	0.36	0.25	0.25	0.25	0.25	0.20	0.53	1.00



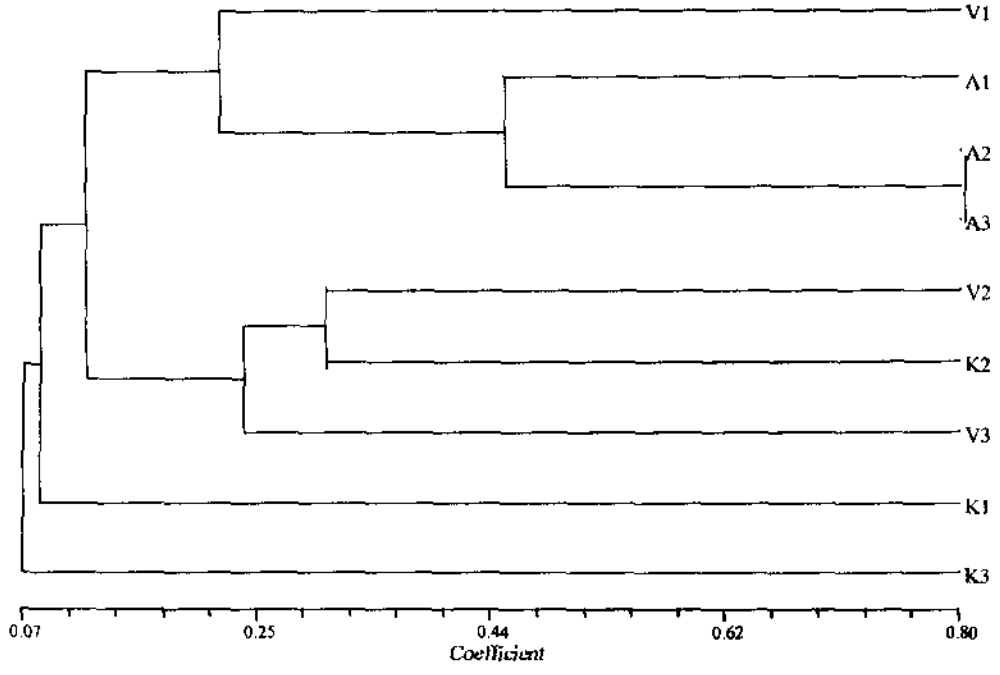
7. Genetic similarity as DICE coefficients and dendrogram based on the RAPD profiles generated by the primer OPF-8

	V1	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.40	1.00							
V3	0.36	0.55	1.00						
K1	0.60	0.20	0.55	1.00					
K2	0.77	0.62	0.57	0.46	1.00				
K3	0.36	0.36	0.50	0.36	0.29	1.00			
A1	0.67	0.44	0.20	0.44	0.67	0.20	1.00		
A2	0.67	0.44	0.20	0.44	0.67	0.20	1.00	1.00	
A3	0.67	0.44	0.20	0.44	0.67	0.20	1.00	1.00	1.00



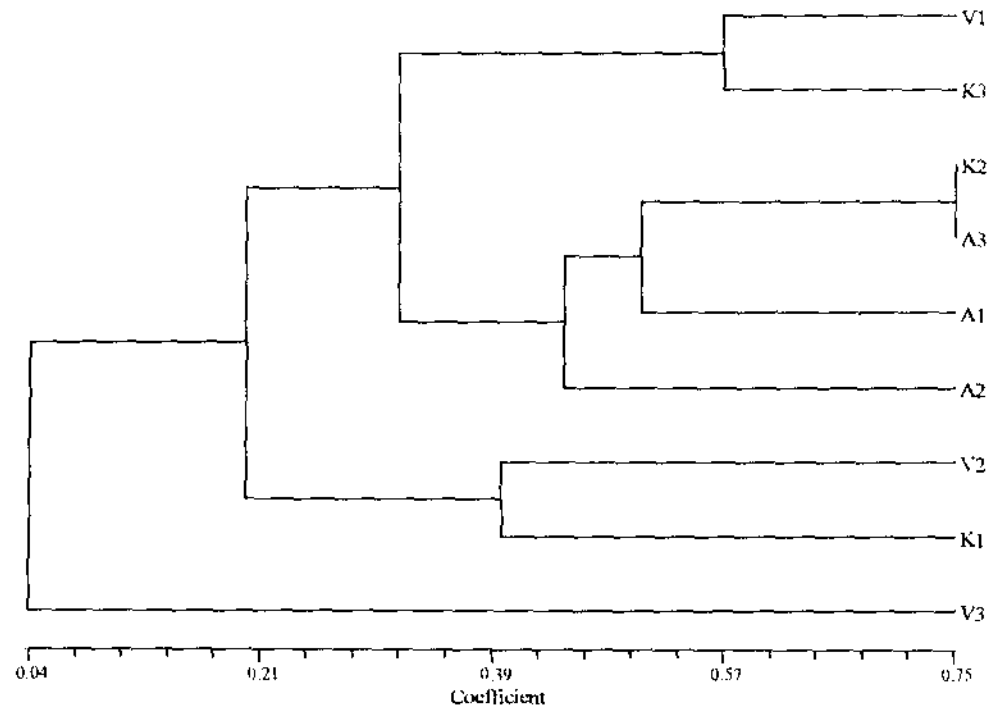
8. Genetic similarity as DICE coefficients and dendrogram based on the RAPD profiles generated by the primer OPP-2

	V1	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.20	1.00							
V3	0.00	0.29	1.00						
K1	0.22	0.00	0.00	1.00					
K2	0.00	0.31	0.20	0.17	1.00				
K3	0.00	0.00	0.00	0.00	0.17	1.00			
A1	0.18	0.00	0.00	0.20	0.14	0.40	1.00		
A2	0.33	0.17	0.00	0.00	0.40	0.00	0.46	1.00	
A3	0.15	0.15	0.00	0.00	0.38	0.00	0.43	0.80	1.00



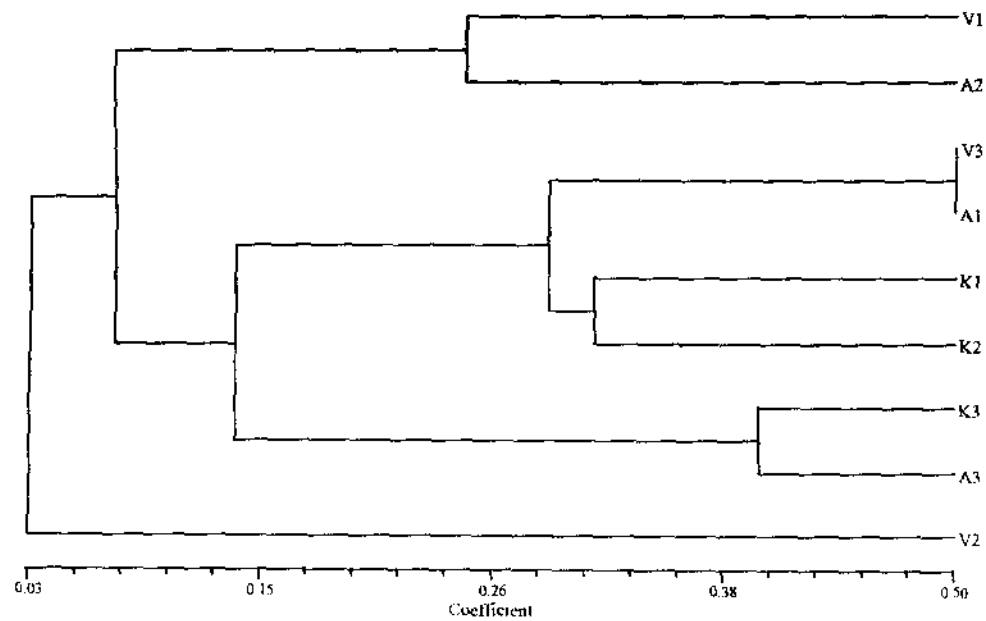
9. Genetic similarity as DICE coefficients and dendrogram based on the RAPD profiles generated by the primer OPP-10

	V1	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.00	1.00							
V3	0.00	0.00	1.00						
K1	0.00	0.40	0.00	1.00					
K2	0.29	0.00	0.29	0.17	1.00				
K3	0.57	0.50	0.00	0.33	0.40	1.00			
A1	0.33	0.29	0.00	0.36	0.44	0.44	1.00		
A2	0.00	0.25	0.00	0.33	0.40	0.20	0.44	1.00	
A3	0.40	0.00	0.00	0.20	0.75	0.50	0.57	0.50	1.00



10. Genetic similarity as DICE coefficients and dendrogram based on the RAPD profiles generated by the primer OPAH-8

	V1	V2	V3	K1	K2	K3	A1	A2	A3
V1	1.00								
V2	0.00	1.00							
V3	0.00	0.00	1.00						
K1	0.00	0.22	0.20	1.00					
K2	0.27	0.00	0.31	0.32	1.00				
K3	0.29	0.00	0.00	0.00	0.14	1.00			
A1	0.00	0.00	0.50	0.43	0.24	0.22	1.00		
A2	0.25	0.00	0.00	0.17	0.13	0.00	0.00	1.00	
A3	0.00	0.00	0.00	0.20	0.00	0.40	0.50	0.00	1.00



MOLECULAR CHARACTERISATION OF *Ralstonia solanacearum* (Smith) YABUUCHI *et al.* CAUSING BACTERIAL WILT IN SOLANACEOUS VEGETABLES

By

DEEPA JAMES

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture

KERALA AGRICULTURAL UNIVERSITY

Department of Plant Pathology

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR-680656

KERALA, INDIA

2001

ABSTRACT

Bacterial wilt incited by *Ralstonia solanacearum* is one of the most devastating diseases of solanaceous vegetable crops in Kerala. Crop losses due to the incidence of this disease may go upto 100 per cent. Existence of different strains, races and biovars has been responsible for breaking down of resistance of varieties evolved through breeding programmes. In view of wide variability, a study was undertaken to characterise the isolates of *R. solanacearum* collected from three different agro climatic zones of Kerala at molecular level.

Nine isolates of *R. solanacearum* collected from three different locations from brinjal, chilli and tomato were used in the study. These were isolated, purified and maintained in sterile distilled water at room temperature.

Inoculation techniques were standardised in brinjal, chilli and tomato plants for assessing the virulence and aggressiveness of the isolates. Virulence and aggressiveness of the isolates were studied on respective host plants and found them highly varying. Vellanikkara and Kumarakom isolates could cross inoculate, whereas Ambalavayal isolates did not.

The isolates were characterised by various cultural, morphological and biochemical tests and the variability among them was studied. Biovars, III and IIIA and races, 1 and 3 were identified among the isolates. The isolates were resistant to ampicillin and sensitive to chloramphenicol.

Plasmid DNA profile of the isolates were studied and no difference was found in the plasmid DNA profile of the nine isolates.

Polymorphism among the isolates was studied using RAPD with ten decamer primers. RAPD profiles exhibited great diversity among biovars III and IIIA as well as among race 1 isolates. Race 3 isolates were less polymorphic with certain primers tested. OPF8 yielded a unique band specific to race 3 isolates. Dendrogram obtained from the pooled data of RAPD profiles also showed high genetic similarity between race 3 isolates.

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Restriction analysis could not characterise the isolates since no banding pattern was obtained with restricted DNA. No hybridization signal was detected after Southern hybridization in RFLP.

Curing of plasmid DNA at high temperatures was found unsuccessful. Plasmid profiles of both mucoid and non-mucoid colonies were compared to assess the role of plasmid in EPS production and the plasmid could be observed in both types of colonies. In the latter, a reduction in size of the plasmid was noticed.

Thus the study revealed that great diversity existed among strains of *R. solanacearum* at different locations of Kerala when molecular techniques, especially RAPD was used as a tool.