CHARACTERIZATION OF *Ralstonia solanacearum* (Smith) YABUUCHI *et al.* CAUSING BACTERIAL WILT IN GINGER USING MOLECULAR MARKER

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Characterization of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* causing bacterial wilt in ginger using molecular marker"** is a bonafide record of research work done by me during the course of research and 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 **"Characterization of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* causing bacterial wilt in ginger using molecular marker"** is a record of research work done independently by Mr. P. K. Sambasivam 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 him.

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ABBREVIATIONS

CPBMB	-	Centre for Plant Biotechnology and Molecular Biology
cm	-	centi meter
°C	-	degree Celsius
DNA	-	Deoxyribo Nucleic Acid
et al.	-	and others
g	-	gram
h	-	hour
l	-	litre
LB	-	Luria Bertani
min	-	minutes
ml	-	milli litre
mM	-	milli Molar
µg	-	micro gram
µl	-	micro litre
ng	-	nano gram
OD	-	Optical Density
PCR	-	Polymerase Chain Reaction
RAPD	-	Random Amplified Polymorphic DNA
rpm	-	revolutions per minute
SDS	-	Sodium Dodecyl Sulfate
TZC	-	Triphenyl Tetrazolium Chloride

Introduction

1. INTRODUCTION

Ginger (*Zingiber officinale* Rosc.), one of the oldest known spices, is esteemed for its aroma and pungency. Rhizomes of this plant are used both as spice and in medicine as carminative, digestive and alterative. Its aroma is due to the presence of volatile oils, which contains zingiberene as the main principle. The flavouring and medicinal potential of the crop gives enough scope for value addition.

India is the largest producer, consumer and exporter of ginger in the world. It occupies 83,940 ha with a production of 3,06,960 tonnes, of which 6,580 tonnes, valued at Rs 22.95 crores were exported in the year 2000 – 2001 (Johny and Ravindran, 2002). In India, Kerala occupies the largest area under ginger followed by Orissa and Meghalaya. In Kerala, ginger is cultivated in an area of 14,568 ha with a production of 49,946 tonnes (FIB, 2001). Though Jamaica produces the best quality ginger in the world, the Cochin and Calicut ginger traded from Kerala are equally reputed in the international market.

A number of pathogens are reported to infect ginger causing several diseases resulting in heavy crop losses. Well known among them are *Pythium aphanidermatum* (rhizome rot disease), *Ralstonia solanacearum* (bacterial wilt), *Phyllosticta zingiberi* (Phyllosticta leaf spot), *Pellicularia filamentosa* (thread blight) and *Fusarium oxysporum* f *sp zingiberi* (yellow disease).

Among these diseases, bacterial wilt caused by *Ralstonia solanacearum* is one of the most serious constraints in the production of ginger (Kumar and Sarma, 2000). In India, the disease was first reported from Kerala (Mathew *et al.*, 1979). *R. solanacearum* is known to infect several hundred host plants,

belonging to fifty families and it has the capacity to survive in soil for long periods. *R. solanacearum* exhibits wide variability and it is a complex species, existing as different biovars and races. This often leads to confusion among scientists in grouping of different isolates. Conventional grouping of isolates into different races and biovars is based on their morphological and biochemical characters. This requires frequent isolation, purification, culturing and characterization using biochemical, physiological and cultural tests. These tests are often time consuming and tedious.

Recently, molecular techniques are gaining more importance since these are highly specific, rapid and can detect even small variation among closely related strains. If genetic fingerprint could be developed for each race / biovar, this would help in their rapid identification and also a better understanding of the pathogen.

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

i) Isolation of the pathogen from bacterial wilt-affected plants collected from ginger-growing tracts in Palakkad, Ernakulam, and Wynad districts.

ii) Cultural and biochemical characterization of the isolates.

iii) Molecular characterization using Random Amplified Polymorphic DNA (RAPD) technique and plasmid profile.

Review of Literature

2. REVIEW OF LITERATURE

Almost hundred years have elapsed since Erwin F. Smith first published his reports describing *Pseudomonas solanacearum*, the causal organism of wilt disease. Its initial recognition in many tropical and subtropical areas followed the first cultivation of solanaceous crops such as potato and tobacco in virgin forests. Later, it was found to infect several hundred species belonging to 44 families (Buddenhagen and Kelman, 1964 and Hayward 1991). Smith (1944) noted that *P. solanacearum* could persist for several years in fallowed soil, even in the absence of the vegetation.

Intially, the bacterium was described as *Pseudomonas solanacearum* by E.F.Smith. Later Yabuuchi *et al.* (1992) transferred several species of the rRNA homology group II Pseudomonads including *Pseudomonas solanacearum* to the genus *Burkholderia.* Due to difference in the 16S rRNA gene sequence within *Burkholderia,* a new genus as *Ralstonia* emerged (Yabuuchi *et al.*, 1995).

The earliest report of *P. solanacearum* causing wilt of ginger was made by Orian (1953) from Mauritius. Ishii and Aragaki (1963) noticed the disease in Hawaii. Jamil (1964) observed the disease in Malaya; Hayward *et al.* (1967) in Queensland; Zehr (1969) in Philippines and Chew (1969) in Malaysia.

Mathew *et al.* (1979) made the first authentic report of occurrence of ginger wilt incited by *P. solanacearum* from India. French (1979) reported that Amazon basin in Brazil and Peru as an area of greater diversity for *P. solanacearum.* Gunawardena *et al.* (1980) noticed the occurrence of wilt in Sri Lanka. Moffett *et al.* (1981) found the epiphytic survival of *P. solanacearum* in capsicum leaves for more than 15 days. Granada and Sequeira (1983) reported that bacterium invades

the roots of presumed non-hosts such as bean and maize. Long-term survival was associated with localised or systemic infection without symptom expression.

In India, *Ageratum conyzoides* and *Ranunculus scleratus* showed no symptoms even though the bacterium was readily isolated from surface sterilised roots (Sunaina *et al.*, 1989). Hayward (1991) reported that epiphytic phase was important in the life cycle of the bacterium. He also found that soil acts as vehicle for the spread of bacterium and a high level of wilting occurred in soils previously planted with susceptible plant cultivars. Sharma and Rana (1999) reported that bacterial wilt caused by *R. solanacearum* was observed in 80 per cent of fields surveyed in Himachal Pradesh. In Japan, the disease was reported for the first time in 1995 (Tsuchiya *et al.*, 2002). Chakrabarti *et al.* (2002) reported that *R. solanacearum* in advanced stage of wilting, the wild types spontaneously generated avirulent variants (phenotypic conversion). The average rate of spontaneous reversion was around three per cent, which increased significantly under stress conditions. This was found essential for long-term survival.

2.1 ISOLATION AND MAINTENANCE OF THE PATHOGEN

2.1.1 Isolation of *R. solanacearum*

Okabe (1949) developed a selective medium to differentiate weakly pathogenic or avirulent mutants from fludial wild type. Kelman (1954) modified the medium by adding triphenyl tetrazolium chloride (TZC). The most common mutant formed were round, butyrous, deep red colony with a narrow bluish border and in contrast the normal or wild type formed an irregularly round fludial white colony with a pink centre. The same TZC medium was also used by several

other workers for isolating the pathogen (Husain and Kelman, 1958; Khan *et al.*, 1979; Nayar, 1982; He *et al.*, 1983; Swanepoel and Young, 1988; Prior and Steva, 1990; Jyothi 1992; Paul 1998 and James, 2001).

Okabe (1969) developed potato dextrose agar with crystal violet (PDA CV). He recovered 10^4 cells of *P. solanacearum* per ml of soil suspension. A selective medium was developed by Karganilla and Buddenhagen (1972) that reduced the background population by 90 per cent; but recovery of *P. solanacearum* below 10^3 cells/g oven dried soil was highly variable. The medium consisted of simplified basal material plus anti microbial compounds. Nesmith and Jenkins (1979) developed a new selective medium (FSM) in which the anti- microbial compound was added at the time of isolation. Colonies of *P. solanacearum* on the FSM were similar in appearance to those observed on TZC. Penicillin G, tetrazolium chloride and vancomycin were used as the principal inhibitors of Gram positive bacteria, bacteracin, tyrothricin and chloromycetin were used as broad spectrum antibiotics. Tetrazolium chloride was used as the major inhibitor for Gram negative soil borne bacteria. The use of yeast extract in FSM and an elemental stock solution favoured the growth of *P. solanacearum* and the colonies were recognised within 36 to 48 h instead of 72 h required previously. The C:N ratio was changed by reducing dextrose to 4 g/l thus reducing the numbers and growth rate of actinomycetes on dilution plates. Mathew *et al.* (1979) isolated the pathogen from ginger on potato dextrose agar medium. The colonies appeared small, circular, smooth and slimy.

Chen and Echandi (1982) developed TZC CP medium by adding chloramphenicol (10 µg/ml) to TZC basal medium. The medium was found effective by reducing 75 – 95 per cent contamination by other bacteria and fungi. This medium was found to detect only the bacteriocin producing strains. Several

factors like incubation period, concentration and quantity of agar, concentration of indicator stain act as limiting factor.

Granada and Sequeira (1983) developed SM_1 and SM_2 media to isolate *P. solanacearum* from artificially and naturally infected soils. Crystal violet, thimerosal, polymyxin B sulfate, tyrothricin and chloromycetin were used as antimicrobial compounds to reduce bacterial contamination. To reduce fungal growth, cycloheximide or chlorothalonil was added. In case of SM_2, norobiocin and nalidixic acid were substituted for tyrothricin and chloromycetin. The plating efficiency ranged from 80 – 100 per cent of that on TZC medium due to surfactant characteristics of antibiotics polymyxin and tyrothricin. The selective medium was stored for about one month at 4° C without noticeable reduction in efficiency. Hara and Ono (1983) developed Hara and Ono' s medium (HOM), which was found 10 - 100 times superior to other media including EBM, MBM, FSM and PDCVA. Engelbrecht (1994) compared a modified form of a selective medium developed by Graham Lloyd (SMSA) with the original medium (GL) and SM_1 medium. He found GL as efficient but failed to reduce soil saprophyte contamination.

Hara *et al.* (1995) developed the improved (HOM) named PCCG which showed higher selectivity and similar sensitivity compared to the original HOM. By using PCCG, *R. solanacearum* was detectable from artificially infected soil containing as little as 10^2 cells/g of dry soil. The appearance of colonies on the medium was similar to TZC and HOM.

2.1.2 Maintenance of *R. solanacearum*

After isolation of a pathogen, it is essential to maintain the organism for further studies. To maintain bacterial cultures there are several methods such as overlaying cultures with mineral oil, lyophilisation (freeze drying), storage in sterile water etc.

R. solanacearum was earlier maintained on PDA slants covered with sterile mineral oil (Kelman and Jenson, 1951; Winstead and Kelman, 1952). By this method, the culture remained viable for four years. Kelman (1954) found that pathogenic cultures could be recovered from isolates for as long as $6^{1}/_{2}$ years under mineral oil. Husain and Kelman (1958) maintained stock cultures by suspending five loopful of bacterial culture in 5ml of sterile distilled water and storing at 25°C. Similar method was also followed by other workers. (Kelman and Person, 1961; Khan *et al.*, 1979; Granada and Sequeira, 1983; Swanepoel and Young, 1988). Stock cultures in sterile water were also maintained at room temperature (He *et al.*, 1983; Prior and Steva, 1990; Mathew *et al.*, 2000 and James, 2001). Kumar *et al.* (1993) maintained the above type of cultures under refrigerated conditions (4°C).

2.1.3 Pathogenicity

Pathogenicity is the capacity of a pathogen to induce malfunctions or interfere with the physiological activities of the plant.

Okabe (1949) while studying *P. solanacearum* in Japan related colony characters with pathogenicity. He noted that weakly pathogenic or avirulent mutants differed in colony morphology from the fluidal wild type.

Later, Kelman (1954) related pathogenicity with colony characters on TZC medium. He conducted inoculation tests on tomato seedlings and found that butyrous red colonies were either weakly pathogenic or non pathogenic, while cultures from fluidal white colonies with pink centre were highly pathogenic. Similar work was done by James (2001) while studying the isolates infecting solanaceous vegetables in Kerala.

2.1.3.1 Methods of inoculation

To test the pathogenicity of *R. solanacearum*, several inoculation techniques have been attemptd by earlier workers. Winstead and Kelman (1952) tested four different inoculation methods to test the pathogenicity of *R. solanacearum* strains infecting solanaceous crops. Among the different methods used like stem puncturing, root injury, root dipping and soil drenching, they found stem puncturing to be the most effective method. This method was later followed by several other workers (Husain and Kelman, 1958; He *et al.*, 1983 and Prior and Steva, 1990). Several workers also followed root inoculation (Khan *et al.*, 1979; Swanepoel and Young, 1988; Paul, 1998 and James, 2001). James (2001) found leaf clipping as effective method to inoculate chilli and tomato. Mathew (2001) also reported leaf clipping as an effective method for the inoculation of the bacterial pathogen of solanaceous vegetables.

2.2 HYPERSENSITIVITY REACTION

Hypersensitivity is the process, in which rapid death of the cells in the vicinity of the invading pathogen occurs. It is the sign of high resistance approaching immunity. Based on hypersensitivity reaction, the isolates can be

grouped into races. However, earlier classification of the bacterium into races was based on either host range or geographical distribution.

Buddenhagen *et al.* (1962) grouped different isolates of *P.solanacearum* into three races based on host range.

Race 1: Affecting tobacco, tomato, many solanaceous and other weeds, certain diploid bananas.

Race 2: Affecting triploid bananas, heliconia or both.

Race 3: Affecting potato and tomato.

Lozono and Sequeira (1970) grouped *P. solanacearum* isolates into three races based on hypersensitive reaction on tobacco by leaf infiltration technique.

Race 1: No visible symptom on inoculated leaves after 24 h, dark brown necrotic lesion surrounded by a yellow halo appeared after 36 h. By 60 h, the bacteria had invaded the adjoining tissues and vascular elements. After eight days extensive wilting yellowing and necrosis of the leaf tissue resulted.

Race 2: Produced water soaked area after 10 to 12 h followed by light yellowing. By 60 h the affected area become papery and dried up. A typical hypersensitivity reaction occurring on tobacco.

Race 3: Yellowish discoloration of the infiltrated areas by 48 h after inoculation.

Based on host range, geographical distribution and ability to survive under different environmental conditions Persley *et al.* (1985) grouped the bacterial pathogen into five races.

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

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

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

Race 4 (Ginger strain): from Philippines.

Race 5 (Potato strain): from China.

In China, most of the strains of *P. solanacearum* were found to belong to race 1 (He *et al.*, 1983). Prior and Steva (1990) reported 23 out of 24 strains collected from French West Indies belonged to race 1 and the one exception as race 3. Akiew and Hybe (1993) reported race 2 strain infecting heliconia in Australia. Rahman *et al.* (1996) reported race 1 infecting chilli, eggplant, potato and tomato. Samadder *et al.* (1998) identified *R. solanacearum* affecting aubergine, tomato, potato and chilli collected from West Bengal as race 1. In Kerala, Mathew *et al.* (2000) identified race 1 infecting solanaceous vegetable. James (2001) identified race 1 and race 3 infecting the above vegetables. Sinigagha *et al.* (2001) identified race 1 infecting tomato, potato and pepper in Brazil. Similar race was also identified in United States infecting tobacco (Robertson *et al.*, 2001).

2.3 HOST RANGE OF THE PATHOGEN

Many of the pathogens have the capability to infect plants other than their own host. This enables the pathogen for long term survival.

Ishii and Aragaki (1963) reported that ginger isolates were capable of causing wilt on chilli. Digat and Escudie (1967) demonstrated that the pathogen isolated from tomato, brinjal and chilli were virulent on solanaceous members.

He *et al.* (1983) studied cross inoculation of strains from six major hosts, viz., brinjal, tomato, capsicum, potato, peanut and tobacco. Rapid wilting was noticed on eggplants on inoculation of all strains. Tomato was susceptable to all strains except that obtained from mulberry. Potato was also susceptable to most strains but the rapidity of wilting varied. Capsicum, peanut and tobacco showed marked differences in their reactions to specific strains.

Swanepoel and Young (1988) studied the pathogenicity of South African *P. solanacearum* strains on potato, tomato, tobacco, eggplant, capsicum, peanut, sunflower and large thorn apple. Potato strains were virulent to all hosts except sunflower, tobacco and peanut. Strains from tomato and tobacco were virulent to all the eight hosts.

Prior and Steva (1990) classified *P. solanacearum* strains based on cross infectivity and host reaction on tomato, brinjal, potato, pepper, peanut, bean and tobacco.

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.

In India, Patel *et al.* (1952) and Hingorani *et al.* (1956) reported that the potato isolates could not infect tobacco and chilli plants. Devi (1978) observed that chilli strain caused high degree of wilting in tomato and brinjal and that of brinjal and tomato isolates were capable of cross infecting each other. However, chilli isolate failed to produce disease on tobacco and potato whereas brinjal and tomato could be one of the hosts of the pathogen (Khan *et al.*, 1979 and Jyothi,

1992). Jyothi (1992) reported that the isolates of chilli caused high degree of wilting on ginger. Rani (1994) found that ginger isolates produced typical wilting in chilli, low incidence on tomato and no symptom on brinjal. Paul (1998) found that tomato, brinjal and chilli isolates were cross infectable producing symptoms at varying intensities. Similar results were also obtained by James (2001).

2.4 CHARACTERISATION OF THE PATHOGEN

Isolates of *R. solanacearum* were characterized and grouped based on cultural, biochemical and molecular tests.

2.4.1 Cultural characterization

Based on the growth habits of pathogen on culture medium, they are characterized. Colony morphological features like shape, size, colour, elevation etc are used as criteria for cultural characterization.

Smith (1896) made the first report on shape and size of *P. solanacearum* as non-spore forming, non-capsulate, gram negative, small rods with polar flagella.

Okabe (1949) was the first to describe variation in colony morphology and he related it with pathogenicity. He grouped them as

1) F - wild type with fluidal, irregular milky colony.
2) OP- opalescent circular homogenous.
3) C- circular, light brownish, striated.
4) SS- Pale, fluorite green with cream coloured centre.

Later, Kelman (1954) observed three main colony types

1) Normal or wild type - Irregular, round, fluidal, smooth and opaque.
2) Mutant type - Uniformly round, butyrous, smooth and translucent.
3) Butyrous type - Rough surfaced.

Similar colony types were also noticed by other workers (Husain and Kelman, 1958; He *et al.*, 1983; Mathew and Nayar, 1983; Prior and Steva, 1998 and Paul, 1998). Mathew *et al.* (1979) observed the colonies as small, circular, white, smooth and slimy while isolating the ginger strain on potato dextose agar medium. On TZC medium he observed pink centred colonies. Khan *et al.* (1979) made a comparative study on the morphological and cultural characters of the isolates from brinjal, chilli, tomato and potato on TZC medium. Similar studies were also done by other workers (Paul, 1998; Mathew *et al.*, 2000 and James, 2001). Rani (1994) observed the ginger strain on TZC medium as circular, smooth, raised, fluidal and slimy colonies with a light pink centre.

2.4.2 Biochemical characterization

R. solanacearum isolates are usually characterized and grouped based on biochemical tests.

2.4.2.1 Gram reaction

It was the foremost test to be done to characterize any pathogenic bacteria. Suslow *et al.* (1982) developed a simple test, i.e. solubility in three per cent KOH solution to study the Gram reaction.

2.4.2.2 Enzyme activity

Kovacs (1956) divided aerobic or facultatively anaerobic bacteria into two groups based on the presence or absence of the cytochrome oxidase enzyme. He grouped them as oxidase positive and negative. Catalase activity was also detected in *P. solanacearum*.

Thornley (1960) used arginine dihydrolase reaction to differentiate *Pseudomonas* from other Gram negative bacteria. He found *P. aeruginosa* and *P. fluorescens* as arginine dihydrolase positive while *P. syringae* and *P. solanacearum* as negative as they are unable to produce ammonia from arginine. Similar biochemical tests were carried out by other workers (He *et al.*, 1983; Swanepoel and Young, 1988; Prior and Steva, 1990; Jyothi, 1992; Rani, 1994; Paul, 1998; Mathew *et al.*, 2000; James, 2001 and Mathew *et al.*, 2002).

2.4.2.3 Nitrate metabolism

Hayward *et al.* (1990) found variation in nitrate metabolism among the biovars. Most of the strains of *P. solanacearum* were found reducing nitrate either with or without producing gas (He *et al.*, 1983; Swanepoel and Young, 1988; Prior and Steva, 1990; Mathew *et al.*, 2000; James, 2001 and Mathew *et al.*, 2002).

2.4.2.4 Glucose oxidation test

Based on glucose metabolism Hugh and Leifson (1953) differentiated bacterial strains as oxidative and fermentative groups. He found *P. solanacearum*

as oxidative. He *et al.* (1983) tested oxidative and fermentative metabolism of *P. solanacearum* strains from China. Swanepoel and Young (1988) tested South African strains and got positive results. Similar work was also carried out by other workers (Rani, 1994; Paul, 1998; James, 2001 and Mathew *et al.*, 2002).

2.4.2.5 Utilization of disaccharides and hexose alcohols

Different isolates of *R. solanacearum* were grouped into biovars based on the utilization of disaccharides and hexose alcohols.

Hayward (1964) classified *P. solanacearum* isolates from different host plants and different areas into four biotypes based on the capacity to utilize disaccharides like lactose, maltose, cellobiose and hexose alcohols like mannitol, sorbitol and dulcitol.

Biotype 1: Unable to oxidize any groups.
Biotype 2: Oxidizes disaccharides alone.
Biotype 3: Oxidizes both disaccharides and hexose alcohol.
Biotype 4: Oxidize hexose alcohol alone.

Later He *et al.* (1983) identified a new biotype 5 to group the isolates from China, which oxidised mannitol but not sorbitol or dulcitol.

Most of the strains affecting solanaceous vegetables belongs to biovar III (Hayward, 1964; He *et al.*, 1983; Prior and Steva, 1990; Paul, 1998; Mathew *et al.*, 2000 and James, 2001).

In Kerala, Devi (1978) classified all the isolates obtained from tomato, brinjal and chilli as biovar IV. Jyothi (1992) characterized *P. solanacearum* from

ginger as biovar III. Paul (1998) identified tomato and chilli isolates as biovar III and brinjal isolate as biovar V. Mathew *et al.* (2000) and Mathew (2001) reported biovar III, III A and V infecting brinjal, chilli and tomato. James (2001) reported biovar III infecting tomato, brinjal and chilli and biovar III A infecting chilli and tomato. Mathew *et al.* (2002) identified biovar III and III A infecting ginger.

2.4.2.6 Intrinsic antibiotic resistance pattern

Among the various control measures, use of antibiotics was found to be effective method to control bacterial disease. Attempts were made by earlier workers to identify suitable antibiotics inhibiting *Ralstonia.*

Moorgan and Goodman (1955) found aureomycin and terramycin to be effective to inhibit *P. solanacearum.* Hindaka and Murano (1956) found Streptomycin to be inhibitive against the pathogen. Similar findings were also reported by other workers (Chakrabarti and Rangarajan, 1966; Farag *et al.*, 1986 and Gunawan, 1989). Samuel (1980) found that ginger strain was sensitive to ambistryn-S, agrimycin-100, chloromycetin, tetramycin and streptocycline at 250 ppm. He *et al.* (1983) and Prior and Steva (1990) reported that several strains of *R. solanacearum* were susceptable to ampicillin, but resistant to chloramphenicol. Jyothi (1992) found chloromycetin and streptocycline to be inhibitive against chilli isolates. Rani (1994) found ambistryn and chloromycetin at 1000 ppm to be effective against ginger isolate. James (2001) found that isolates from solanaceous vegetable were resistant to ampicillin but sensitive to chloramphenicol.

2.4.3 Molecular Characterization

Recently several DNA markers have been developed to study variation at DNA level among the natural population.

2.4.3.1 Plasmid profile

Plasmids are the extrachromosomal DNA molecules replicating autonomously and capable of existing in multiple copies in the bacterial cell. In case of several pathogenic bacteria, they govern the important traits like virulence, toxin production, enzymes, antibiotics resistance and host range.

Different strains of *R. solanacearum* may harbour one or two plasmids, but most of the strains contain only a single plasmid. Morales and Sequeira (1985) reported that 22 out of 39 strains of *P. solanacearum* harboured one or two plasmids with relative masses ranging from five to more than 500 mega daltons. Elumalai and Mahadevan (1997) found Indian strains to harbour one to three plasmids. James (2001) observed a single plasmid in *R. solanacearum* isolates of solanaceous vegetables.

Birnboim and Doly (1979) developed a rapid alkali lysis procedure for screening recombinant plasmid DNA. Kado and Liu (1981) extracted plasmid DNA by alkaline lysis of the cells followed by phenol chloroform extraction. Comai and Kosuage (1982) modified the above method by lysing the cells directly in the wells formed in agarose gels.

Morales and Sequeira (1985) modified the method of Kado by replacing SDS with three per cent sarcosyl in lysing solution. Boominathan and Mahadevan (1988) developed isolation procedure for mega plasmids by slightly modifying the above methods.

2.4.3.2 PCR based RAPD

PCR

Polymerase Chain Reaction (PCR) was discovered by Kary Mullis in 1985 (Saiki *et al.*, 1988). The process consists of amplification of a DNA fragment into millions of copies using a short DNA oligomer as a primer.

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. Fernandez *et al.* (1996) characterized *R. solanacearum* isolates from different plants like hibiscus, tomato, sugarcane, capsicum and coffee using PCR technique. Opina *et al.* (1997) developed a novel method to produce species and strain specific probes and primers for identifying *R. solanacearum*. Seigner (1997) found PCR to be a reliable and sensitive method to detect even low level infection in potato tubers. Ito *et al.* (1998) combined PCR and semisensitive medium technique to detect viable cells of *R. solanacearum* in soil.

Smith *et al.* (1998) used rep PCR to detect genetic diversity among the isolates of European origin. Similar technique was also carried out by other workers (Hartung *et al.*, 1998; Seal *et al.*, 1999 and Boudazin *et al.*, 1999). Roncal *et al.* (1999) developed primers P759/P760 for rapid identification of the pathogen.

Lee and Wang (2000) developed specific primers for detection of *R. solanacearum* in soil samples. Weller *et al.* (2000) developed a fluorogenic (Taq Man) PCR assay to detect the pathogen. Maciel *et al.* (2001) used T_3A and T_5A primers to detect biovar 1 or 2 infecting potato. Robertson *et al.* (2001) used rep PCR to generate fingerprints of *Ralstonia* in United States. Horita and Tsuchiya (2001) used rep PCR to characterize Japanese strains. Glick *et al.* (2002) used multiplex polymerase chain reaction assay to detect *R. solanacearum* infection in geranium.

RAPD

Due to complexicity of the long oligonucleotide primers, recently single short oligonucleotide sequences are being used as primers to amplify random sequences from a complex DNA template.

Opina *et al.* (1997) differentiated three genetically diverse strains belonging to biovars II, III and IV using RAPD technique. Ito *et al.* (1998) used RAPD analysis to detect genomic diversity of 18 isolates in Japan. Salava *et al.* (1998) used 30 synthetic decamer oligonucleotide primers to identify various plant pathogenic bacteria like *Xanthomonas spp., Clavibacter spp., Erwinia amylovora and R. solanacearum.* Thwaites *et al.* (1999) used RAPD and rep PCR to detect genetic relationship between isolates infecting *Musa spp.*

Recently Lee and Wang (2000) used RAPD method to design the specific primers for detecting *R. solanacearum* in soil samples by PCR. Kumar and Sarma (2000) used RAPD to characterize 18 isolates of *R. solanacearum* infecting ginger, tomato, potato, chilli and chromolaena. James (2001) used RAPD technique to characterize nine isolates of *R. solanacearum* infecting solanaceous

vegetable in Kerala. She reported that a 1.45kb fragment obtained through RAPD using OPF 8 primer could differentiate race 3 from others.

Materials and Methods

3. MATERIALS AND METHODS

The experiments for the present study were conducted at the Department of Plant Pathology and Centre for Plant Biotechnology and Molecular Biology in the College of Horticulture, Vellanikkara during the period January 2002- August 2003.

3.1 COLLECTION OF DISEASED SAMPLE

Ginger plants showing wilt symptoms were collected from ginger growing tracts in Palakkad, Ernakulam and Wynad districts. Ooze test was done to confirm bacterial wilt. An oblique sharp cut was made at the basal portion of the pseudostem and the cut end was placed in a test tube containing sterile water. The tube was kept undisturbed and observed for streaming out of ooze from the cut end. A total of 15 samples, five from each district were collected for the experiment (Table 1).

3.2 ISOLATION AND MAINTENANCE OF THE PATHOGEN

The diseased plants collected from the fields were brought to the laboratory, washed under tap water to remove the soil particles and air dried. The pseudostem of diseased plant of length 10-15 cm was first surface sterilized with 70 per cent ethyl alcohol and then cut into pieces. These were then washed with three changes of sterile water and placed in the test tubes containing 5 ml sterile water. The tube was left as such for 10 minutes to get a turbid suspension. The pathogen was isolated on triphenyl tetrazolium chloride (TZC) medium (Appendix) using spread plate technique. A loopful of bacterial suspension was diluted in 100 ml sterile water and from this 1 ml was transferred to 9 ml water blank. This was diluted serially upto 10^{-6} dilutions. About 1 ml of inoculum from the last two dilutions were spread plated on TZC medium contained in Petri plates and incubated at 28 ± 2°C for 48 h. Three to five characteristic single colonies were selected and suspended in test tubes

Table 1. Details of *R. solanacearum* isolates used for characterization

Isolates	Locations	Districts
P1	Kairady	Palakkad
P2	Kappancherry	Palakkad
P3	Payangodu	Palakkad
P4	Kullalmannam	Palakkad
P5	Nenmara	Palakkad
E1	Kottapadi	Ernakulam
E2	Plamudi	Ernakulam
E3	Kunnumpuram	Ernakulam
E4	Muthathupara	Ernakulam
E5	Nedungappara	Ernakulam
W1	Ambalavayal	Wynad
W2	Kalpetta	Wynad
W3	Vaduvanchal	Wynad
W4	Sulthanbathery	Wynad
W5	Cheeral	Wynad

containing 5ml sterile water. The tubes were stored at 4°C and used for further studies (He *et al.*, 1983).

3.3 PATHOGENICITY

3.3.1 Methods of inoculation

Five different inoculation methods viz., pseudostem inoculation, leaf clipping, leaf axil inoculation, soil drenching and rhizome inoculation were tried with P1 isolate. The best method was identified based on Per cent Wilt Incidence (PWI) and Incubation Period (IP).

$$\text{Per cent Wilt Incidence (PWI)} = \frac{\text{No of plants wilted}}{\text{Total no of plants inoculated}} \times 100$$

Incubation Period (IP) = No of days taken by the inoculated plants to produce visible symptoms.

3.3.2 Pathogenicity

Pathogenicity of the isolates was tested on two-month-old healthy ginger plants of Maran cultivar grown in pots. Fresh bacterial ooze collected from wilted plants having a concentration of $OD_{600} = 0.3$ was used for inoculation. The inoculated plants were kept under observation for symptom expression and then the pathogenicity of the isolates was established.

3.4 HYPERSENSITIVITY REACTION ON CAPSICUM

Hypersensitivity reaction of capsicum to different isolates of *R. solanacearum* was studied. Thirty-day-old capsicum plants grown in plastic cups were used. About 0.5 ml of standard bacterial inoculum was infiltrated into the intercostal region on the undersurface of the capsicum leaves using plastic disposable syringe without needles. The inoculated plants were observed for the symptoms on the infiltrated areas. The isolates were grouped into races based on the criteria of Lozano and Sequeira (1970), as detailed bellow.

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 plant die.

Race 2: By 10 to 12 h the leaf area will be water soaked with slightly 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.

3.5 HOST RANGE OF THE PATHOGEN

Host range studies were conducted by cross inoculating different ginger isolates on tomato, brinjal, chilli, pumpkin, bitter gourd, snake gourd and ash gourd. Isolates of *R. solanacearum* from tomato, brinjal and chilli were also cross inoculated on ginger. For each treatment, there were five replications. Inoculated plants were kept for 21 days for symptom appearance. Wilt symptoms were recorded using the score chart suggested by Prior and Steva (1990),

Disease score

1- no symptoms
2- inoculated leaf wilted
3- two or three leaves wilted
4- four or more leaves wilted
5- plant dead

Disease index was calculated as suggested by Emping *et al.* (1962).

$$\text{Per cent disease index} = \frac{\text{Sum of all readings per treatment}}{\text{Total no of plants per treatment} \times \text{maximum disease score}} \times 100$$

Per cent wilt index	Category
0	No infection (N)
< 40	Low (L)
40 – 65	Medium (M)
> 65	High (H)

3.6 CHARACTERIZATION OF ISOLTAES

Isolates of *R. solanacearum* were characterized at cultural, biochemical and molecular levels.

3.6.1 Cultural characterization

Cultural characters of the isolates were studied by streaking it on Kelman's TZC medium. Colony characters like size, shape, colour, margin, elevation and mucoid nature of each isolate were observed after 48 h of streaking.

3.6.2 Staining reaction

R. solanacearum comes under the group of Gram-negative bacteria. The isolates were stained to confirm its negative reaction.

3.6.3 Biochemical characterization

3.6.3.1 Solubility in three per cent KOH

At the centre of a sterilized glass slide two drops of three per cent KOH solution was placed and a loopful of bacterial growth was rapidly agitated on it in a circular motion. After five to eight seconds the loop was gently raised and lowered just above the slide surface. Presence of viscous strands between the loop and slide surface indicated positive reaction (Suslow *et al.*, 1982).

3.6.3.2 Kovacs oxidase test

To detect the presence of cytochrome oxidase of the bacteria, ready-to-use oxidase discs from Hi media, Mumbai were used. At the centre of a sterile glass slide a disc was placed and a loopful of inoculum was rubbed to get a purple colour. The time taken for colour development by each isolate was noted (Kovacs, 1956).

3.6.3.3 Catalase test

A few drops of three per cent H_2O_2 were placed at the centre of the sterile glass slide and a loopful of bacterial inoculum was agitated in the solution. Formation of air bubbles indicated the positive reaction (Cappucino and Sherman, 1992).

3.6.3.4 Arginine dihydrolase reaction

For this test Thornley's semisolid medium (Appendix) was used. About 5 ml quantities of the medium were dispensed in 15 ml capacity screw capped test tubes and sterilized in autoclave at 121° C at 15 psi. The tubes after sterilization were kept in vertical position to set at room temperature. The medium was inoculated by stabbing with sterile bacterial loop containing inoculum. The surface of the medium was sealed with 3 ml molten one per cent agar at 45° C and the tubes were incubated for seven days at 28° C. Any colour change was recorded at regular intervals.

3.6.3.5 Oxidation of glucose

For this test Hayward's semisolid basal medium (Appendix) was used. After dissolving the constituents of the medium the pH was adjusted to neutral using 40 per cent NaOH. At neutral pH the medium appeared olivaceous green in colour. Then agar at 0.3 per cent concentration was added and the medium was sterilized.

Glucose solution was prepared separately by adding 1g of glucose to 10 ml of sterilized distilled water and sterilized by tyndallization method. This solution was added to 90 ml of the basal medium before inoculation. About 5 ml of this mixture was dispensed in sterile screw capped test tubes and allowed to solidify. A control was maintained without adding glucose solution to the medium. All the tubes were inoculated with bacterial suspension having concentration of $OD_{600} = 0.3$. The tubes were incubated at room temperature and observations were taken on colour change (Hugh and Leifson, 1953).

3.6.3.6 Gas production from Nitrate

Van den Mooter Glycerol (VMG) medium (Appendix) was used for this test. About 5 ml of the medium was dispensed in screw-capped test tubes and sterilized in autoclave at 121°C at 15 psi. The medium after solidification was stab inoculated with bacterial cultures. The tubes were incubated at room temperature for seven days and observations were recorded for the production of gas (Hayward *et al.*, 1990).

3.6.3.7 Utilization of disaccharides and hexose alcohols

The isolates of *R. solanacearum* were categorised into biovars based on their ability to utilise disaccharides (lactose, maltose and cellobiose) and hexose alcohols (mannitol, sorbitol and dulcitol) as a source of carbon and energy (Hayward, 1964 and He *et al.*, 1983). Hayward's semisolid medium (Appendix) was used for this test (Hayward, 1964). After dissolving the constituents of the medium, the pH of the medium was adjusted to neutral to get olivaceous green colour. Then the medium was sterilised after mixing with agar at 0.3 per cent concentration. Sugar and hexose alcohol solutions were prepared by dissolving 1g in 10 ml of sterile distilled water and were sterilised by tyndallisation. This solution was added to 90 ml basal medium and mixed well by rotation. About 5 ml of this was taken in sterile screw capped tubes and allowed to set at room temperature. A control was maintained for each isolate without the addition of sugar solution to the basal medium. Fifty microlitre of bacterial suspension prepared by dispensing single colony in sterilised water (OD_{600} = 0.3) was used for inoculation. The tubes were incubated at room temperature and observations were taken on colour change.

3.6.3.8 Intrinsic antibiotic resistance pattern

Tryptone Yeast Extract (TY) medium (Appendix) was used to study the intrinsic antibiotic resistance pattern of the bacterial isolates. To the plates containing the medium and suitable concentration of antibiotics (Table 2), 10 µl of dilute bacterial suspension was spotted. A control was maintained without antibiotics. Plates were observed for bacterial growth after 24 h of incubation at room temperature.

3.6.4 Molecular characterization

R. solanacearum isolates were characterized at molecular level using plasmid profile and DNA fingerprinting by RAPD. An attempt was also made to confirm the race classification as reported by James (2001).

3.6.4.1 Plasmid profiling

Plasmid profile of different isolates of *R. solanacearum* was examined. An attempt was also made to characterize the plasmid by transformation to *Escherichia coli.*

3.6.4.1.1 Plasmid DNA isolation

Plasmid DNA was isolated using alkali lysis method (Birnboim and Doly, 1979). The procedure is given bellow

Tubes containing 2 ml LB medium and ampicillin 50 µg/ml were inoculated with a single bacterial colony.

↓

Table 2. Details of antibiotics used for intrinsic antibiotic resistance screening

Antibiotics	Stock conc. (mg/ml)	Solvent	Working conc. (µg /ml)
Ampicillin	50	Water	50 100
Carbenicillin	100	Water	50 100
Chloramphenicol	50	70% Ethanol	150 200
Gentamycin	10	Water	25 50
Kanamycin	100	Water	50 100
Nalidixic acid	100	Water	50 100
Rifampicin	75	Water	50 100
Streptomycin sulphate	75	Water	50 100
Tetracycline	50	70% Ethanol	25 50

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

↓

Centrifugation at 12000 rpm for 8 min

↓

Supernatent removed carefully without disturbing the pellets

↓

Cell pellets resuspended in 100 µl resuspension buffer

↓

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

↓

120 µl of neutralization buffer was 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

↓

To this 200 µl isopropanol added and mixed for 1 min

↓

Centrifugation at 13000 rpm for 1 min to get DNA pellet

↓

70 per cent ethanol (500 µl) DNA wash and air drying

↓

Pellet dissolved in 100 µl sterile water

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

3.6.4.1.2 Agarose gel electrophoresis

Agarose gels separate DNA on the basis of size by migration of DNA through a matrix under the influence of an electric field.

a) Preparation of agarose gel

The two open sides of the gel-casting tray were sealed with cellotape and placed on a uniform level surface

↓

Agarose at 0.8 percent conc. in 1X TAE buffer was prepared, boiled and allowed to cool to 45°C

↓

Ethidium bromide at 0.5 µg/ml conc. was mixed with gel and poured into the tray having comb in position (gap should be maintained between the comb and tray so as to form a well on the gel)

↓

After solidification, the gel was placed for electrophoresis.

b) Electrophoresis

After removing the cellotape the gel along with tray was placed into the horizontal electrophoresis tank (Hoefer, USA)

↓

Electrophoresis buffer (1X TAE) was added to a level until it covered the gel surface

↓

DNA sample after mixing with loading dye in the ratio of 1:1 was loaded into the wells. The well must be placed near the negative terminals

↓

Marker λ DNA digested with *Eco* R1 and *Hind* III was loaded to determine the size of unknown DNA sample

↓

Power supply with constant voltage of 120 volts was applied to cathode and anode terminals.

When the loading dye reached 2/3 rd distance, the gel was taken and viewed under UV transilluminator. Then the image was documented using Alpha Imager 1200 (Alpha Innotech Corporation, USA).

3.6.4.1.3 Plasmid transformation

a) Preparation of competent cells

For competent cell preparation *E. coli* strain DH 5α [F, lac ZAM 15, hsd R17 rec Al, gyr A 96, thi-1, rel A (Sambrook *et al.*, 1989)] was used. .

About 3.5 ml LB medium was inoculated with a loopful of DH5α cells

↓

Grown overnight at 37°C until OD becomes 0.4

↓

Reinoculated in 50 ml medium and incubated for 2 1/2 h until OD becomes 0.6

↓

Kept in ice for 15 min

↓

Centrifuged at 3500 rpm for 10 min at 4°C

↓

Cells washed with 10 ml of 100 mM $CaCl_2$

↓

Centrifuged at 5000 rpm for 10 min at 4°C

↓

Resuspended in 2 ml of 100 mM $CaCl_2$

↓

Kept in ice for 12-18 h

↓

About 400 µl chilled 100 percent glycerol was added and mixed well.

↓

The cells were divided into aliquotes of 100 µl, frozen in liquid N and stored at -70°C

b) *Transformation of plasmids into competent cells*

About 10 µl of plasmid DNA was added and mixed with 100 µl of competent cells

↓

Stored in ice for 10 to 40 min

↓

Heat shock at 42°C for 2 min was given

↓

Kept immediately in ice for 5 min

↓

About 250 µl of LB medium was added and incubated at 37°C in a shaker for 1 h

↓

Spread plated on Petri plates containing LB Agar medium, ampicillin 100 µg/ml and nalidixic acid 15 µg/ml

↓

Incubated at room temperature

↓

Plates observed for the growth of colonies.

c) *Antibiotic resistance of transformed cells*

To identify the antibiotic resistance genes located on the plasmid, the transformed *E. coli* cells were streaked on LB medium containing antibiotics used for screening *R. solanacearum* isolates. Plates were observed for colony growth. Presence of plasmid was confirmed by reisolating plasmid from transformed *E. coli* cells.

3.6.4.2 DNA finger printing by RAPD

3.6.4.2.1 Genomic DNA isolation

Genomic DNA was isolated by large preparatory method (Girija, 1999). The process consists of lysing the cells, denaturing proteins and precipitating with sodium acetate and isopropanol/ethanol.

About 50 ml of 2YT medium was inoculated with a loopful of bacterial culture and incubated overnight on a shaker at 200 rpm at 37°C

↓

40 ml culture centrifuged at 4°C for 10000 rpm for 10 min

↓

Removal of supernatant and with one per cent NaCl (10 ml) pellets washed twice to remove exopolysaccharides

↓

Pellets dissolved in 8.75 ml TE buffer

↓

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

↓

Chloroform–isoamyl alcohol mixture (10 ml) added and mixed for 5 min

↓

Centrifugation at 10000 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 at 10000 rpm at 4°C for 10 min after adding and mixing chloroform-isoamyl alcohol mixture (10 ml)

↓

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

↓

DNA thread spooled out into a fresh tube

↓

DNA pellet washed with 70 per cent ethanol at 10000 rpm for 10 min

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

3.6.4.2.2 DNA Quantification

Ten µl of the DNA extract was diluted to 1.5 ml with sterile water and absorbance read at 260 and 280 nm using UV visible spectronic 20 Genesys 5 spectrophotometer. The ratio of OD_{260}/OD_{280} values between 1.8-2.0 indicates the purity of DNA. Quantity of DNA present in the sample was calculated using the formula

$OD_{260} = 1$ is equivalent to 50µg double stranded DNA /ml of sample

Quantity of DNA present in the sample = $OD_{260} \times 50$ µg/ml.

3.6.4.2.3. Screening of primers for RAPD

A total of 20 primers (OPERON Technologies, USA) belonging to OPF, OPU and OPX series, available at CPBMB were screened for amplification of genomic DNA. From these, the primers giving good amplification were selected and utilized for further characterization.

3.6.4.2.4 RAPD

Genomic DNA of the isolates were amplified by using random, short oligonucleotide primers. For amplification the DNA preparation was diluted to a concentration of 12.5 ng/µl. A 25 µl reaction mix was prepared for each sample as shown in Table 3. A master mix without template DNA was prepared using the reaction mix. From this mix, 23 µl was pipetted into each PCR tube containing 2 µl of template DNA. After mixing in table top centrifuge, the tubes were kept in PCR machine (Peltier Thermal Cycler-200) and the programme was run. The procedure of Demeke *et al.* (1992) was modified and used for amplification of DNA.

Table 3. Components of reaction mix for RAPD

10 x Assay buffer with 15 mM $MgCl_2$	dNTP Mix (10 mM)	Taq DNA polymerase	Primer (10 pmoles)	Template DNA (12.5 ng/µl)	Sterile Milli Q water	Total
5 µl	1 µl	0.2 µl	2 µl	2 µl	14.8 µl	25 µl

Each cycle involves

DNA denaturation at 94°C for 1 min

↓

Annealing of primer to the template DNA at 37.5°C for 1 min

↓

Primer extension at 72°C for 2 min

A total of 45 cycles were programmed. After completion, the amplified DNA product was electrophoresed and the image was documented as indicated earlier.

3.6.4.2.5 Analysis of genetic similarity

The RAPD profiles of the isolates for different primers were compared and DNA bands were scored. The genetic similarity was estimated by computing Jaccards coefficient using NTSYS PC 2.0 software. Dendrogram were drawn using unweighted pair group arithmetic mean (UPGMA) routine.

Per cent polymorphism for individual primer was calculated as follows

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

Results

4. RESULTS

The results of the study on characterization of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* causing bacterial wilt in ginger undertaken at Department of Plant Pathology and Centre for Plant Biotechnology and Molecular Biology are presented below.

4.1 ISOLATION AND MAINTENANCE OF THE PATHOGEN

Ginger plants showing wilt symptom were collected from ginger growing tracts of Palakkad, Ernakulam and Wynad districts (Plate 1). These plants exhibited symptoms like yellowing of leaves along the margin, inward rolling and easy detachment of pseudostem from the soil. Rhizome of the diseased plant shows greyish brown discolouration. A slant cut was given to the pseudostem of the infected plant and it was kept immersed in the sterile water taken in a test tube. Flow of bacterial ooze was observed. The bacteria isolated on TZC medium produced small creamy white colonies with pink centre (Plate 2). The typical colonies suspended in 5 ml sterile distilled water in screw capped vials and stored at 4°C when tested for virulence at regular intervals also found to produce the typical creamy white pink centred (virulent) colonies.

4.2 PATHOGENICITY

4.2.1 Method of inoculation

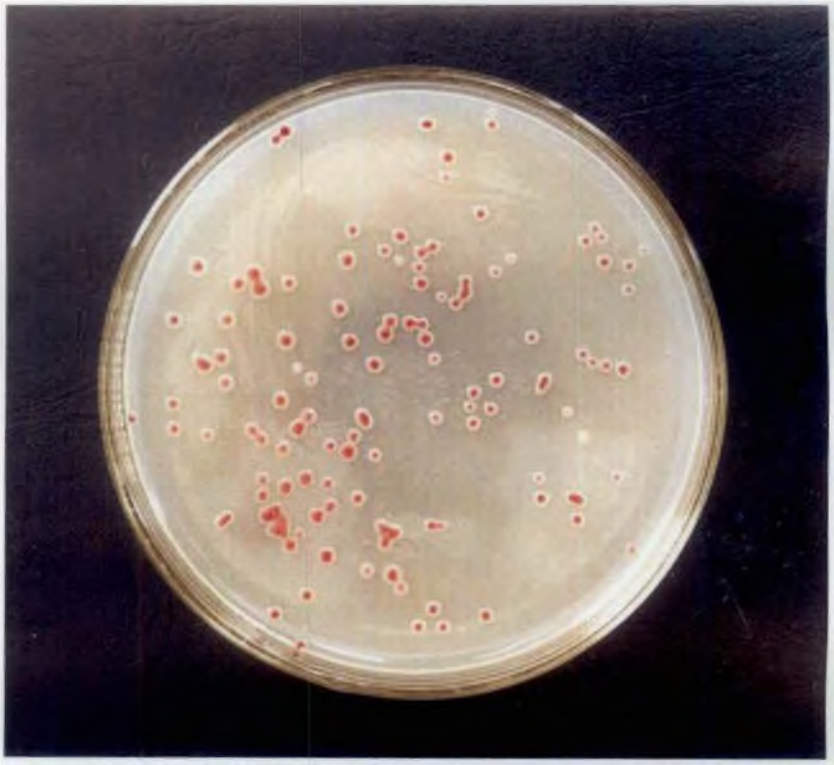
Among the different methods tested, pseudostem inoculation was found to be the best, as it showed early appearance of symptom at five days after inoculation and also recorded cent per cent incidence. In the case of other methods like leaf axil inoculation, rhizome inoculation, and leaf clipping, the symptoms appeared 6, 7, and

Plate 1. Bacterial wilt infected ginger

A - Diseased field

B - Infected plant

Plate 2. Typical colonies of *R. solanacearum* on TZC medium



8 days after inoculation with 60 per cent incidence. Soil drenching method was found to be least effective as evident from the longer time (11 days) taken for the symptom expression and low wilt incidence (40) (Table 4).

4.2.2 Pathogenicity test

Inoculation of different isolates of *R. solanacearum* by pseudostem injection produced typical wilt symptoms on healthy plants (Plate 3). Reisolation of the pathogen from the infected plants also produced the typical colonies of *R. solanacearum* on TZC medium as that of the original culture. Thus the pathogenicity of the organism was established.

4.3 HYPERSENSITIVITY REACTION OF THE ISOLATES

The leaves of capsicum plants infiltrated with bacterial ooze remained symptomless for 24 to 48 h. Water soaked lesions developed after 72 h. By 96 h yellowing was noticed around the lesions (Plate 4), which spread throughout the entire leaf causing defoliation. All the isolates were found to produce the similar symptoms. Based on the above criteria it was found that all isolates belong to race 3.

4.4 HOST RANGE OF THE PATHOGEN

In order to study the host range, *R. solanacearum* causing bacterial wilt in ginger isolates of the pathogen obtained from different locations were inoculated on solanaceous and cucurbitaceous plants. From the results (Table 5) it was evident that all the isolates of the pathogen produced wilt symptoms on tomato and brinjal (Plate 5) within 5 – 7 days recording 80 – 100 per cent wilt index. Other hosts such as chilli, snake gourd, bitter gourd, pumpkin and ash gourd did not produce any wilt

Table 4. Standardization of inoculation methods of *R. solanacearum* in ginger

Methods of inoculation	Per cent Wilt Incidence	Incubation Period (days)
Pseudostem inoculation	100.00	5
Leaf clipping	60	8
Leaf axil inoculation	60	6
Soil drenching	40	11
Rhizome inoculation	60	7

Table 5. Studies on the host range of the *R. solanacearum* isolates infecting ginger

Host plant tested	Isolates used					
	P1		E1		W3	
	Incubation Period (days)	Per cent wilt index	Incubation Period (days)	Per cent wilt index	Incubation Period (days)	Per cent wilt index
Tomato	5	100	5	96	6	92
Brinjal	6	80	6	84	7	88
Chilli	-	0	-	0	-	0
Snake gourd	-	0	-	0	-	0
Bitter gourd	-	0	-	0	-	0
Pumpkin	-	0	-	0	-	0
Ash gourd	-	0	-	0	-	0

Plate 3. Typical wilt symptoms on ginger upon pseudostem inoculation

Plate 4.

Hypersensitive reaction of *R. solanacearum* on capsicum

Plate 5. Host range of *R. solanacearum* infecting ginger

Tomato

Brinjal

symptoms even after 21 days of inoculation indicating that these were non hosts of the pathogen. Only tomato and brinjal were found to be the collateral hosts of the *R. solanacearum* causing bacterial wilt in ginger. *R. solanacearum* isolated from tomato, brinjal and chilli when inoculated on ginger plants, chilli isolate alone produced symptoms after five days of inoculation.

4.5 CHARACTERIZATION OF THE ISOLATES

4.5.1 Staining reaction

All isolates were Gram negative as indicated by red colour upon Gram staining.

4.5.2 Cultural characterization on TZC medium

The cultural characters of the isolates were studied on TZC medium. The pathogen produced creamy white colonies 24 h after inoculation on solid TZC medium. At the centre of the colonies light pink colour developed after 48 h. Colonies of all the isolates were similar in cultural characters. All the isolates produced small, creamy white pink centred circular colonies with entire margin. The colonies were slightly raised and surrounded with fluidal mass.

4.5.3 Biochemical characterization

The isolates were characterized using various biochemical tests (Table 6). All the isolates showed positive reaction for solubility in three per cent KOH, Kovacs oxidase, catalase, glucose oxidation and gas production from nitrate. However, the time taken for colour development in case of Kovacs oxidase test varied with each isolate (Table 7). Isolates from Palakkad showed quick reaction (44 – 58 sec) as

Table 6. Biochemical characteristics of different isolates of *R. solanacearum*

Sl. No	Biochemical tests	Isolates														
		P1	P2	P3	P4	P5	E1	E2	E3	E4	E5	W1	W2	W3	W4	W5
1	Solubility in 3% KOH	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Kovacs oxidase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Arginine dihydrolase reaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	Glucose oxidation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	Gas production from nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

\+ Positive
\- Negative

compared to Ernakulam and Wynad isolates and the P4 isolate recorded the least time (44 sec) for the reaction. None of the isolates produced ammonia from arginine source.

Table 7. Oxidase response of the isolates

Isolates	P1	P2	P3	P4	P5	E1	E2	E3	E4	E5	W1	W2	W3	W4	W5
Time taken (sec)	50	56	58	44	48	64	66	64	68	66	56	62	63	68	66

4.5.3.1 Utilization of disaccharides and hexose alcohols

The isolates of *R. solanacearum* oxidised lactose, maltose, cellobiose, mannitol and sorbitol, but they failed to oxidise dulcitol. However, the utilisation time varied among the different isolates for these sugars. Based on the above criteria all the isolates were categorized as biovar III A (Table 8).

4.5.3.2 Intrinsic antibiotic resistance pattern

All the isolates were found resistant to ampicillin and rifampicin at 100 µg/ml concentrations and sensitive to chloramphenicol and kanamycin at 150 and 50 µg/ml concentrations respectively. The isolates exhibited variability in their sensitivity to other antibiotics tested (Table 9). Based on the data, antibiotic markers were identified for each isolate (Table 10).

4.5.4 Molecular characterization

Isolates of *R. solanacearum* were characterised through plasmid profile and RAPD technique.

Table 8. Utilization of disaccharides and hexose alcohols by isolates of *R. solanacearum*

Sugars	Time (days)	Isolates														
		P1	P2	P3	P4	P5	E1	E2	E3	E4	E5	W1	W2	W3	W4	W5
Cellobiose	1	-	-	-	-	-	±	+	-	+	+	±	+	-	+	±
	2	±	±	±	+	+	+	#	±	#	#	+	#	+	#	+
	3	+	+	+	#	#	#	#	+	#	#	#	#	#	#	#
	4	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#
Lactose	1	±	±	+	±	±	+	+	±	+	+	+	+	±	+	+
	2	+	+	#	+	+	#	#	+	#	#	#	#	+	+	#
	3	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#
Maltose	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	±	±	±	±	-	+	±	-	±	±	+	-	-	-	±
	3	+	+	+	+	±	#	+	±	+	+	+	+	±	+	+
	4	#	#	#	#	+	#	#	+	#	#	#	#	+	#	#
	5	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#
Mannitol	1	+	+	+	+	+	+	+	±	+	+	+	±	±	±	+
	2	#	#	#	+	#	+	#	+	#	#	#	+	+	+	#
	3	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#
Sorbitol	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	±	+	±	±	±	±	+	-	-	-	±	-	+	-	+
	3	+	#	+	+	+	+	#	±	±	±	+	±	#	±	#
	4	#	#	#	#	#	#	#	#	#	#	#	#	#	+	#
Dulcitol	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- \- No change
- ± Yellowing without air bubbles
- \+ Yellowing with few air bubbles
- \# Yellowing with many air bubbles

Table 9. Intrinsic antibiotic resistance patterns of the isolates

Antibiotics	Conc. (µg /ml)	Isolates														
		P1	P2	P3	P4	P5	E1	E2	E3	E4	E5	W1	W2	W3	W4	W5
Ampicillin	50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbencillin	50	-	-	-	-	±	-	-	±	±	±	-	±	±	±	±
	100	-	-	-	-	±	-	-	-	-	±	-	-	±	-	-
Chloramphenicol	150	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	200	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentamycin	25	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	50	-	±	-	±	±	-	±	±	-	±	-	±	±	-	±
Kanamycin	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nalidixic acid	50	±	±	±	±	±	-	±	±	±	-	-	-	±	±	±
	100	-	-	-	-	±	-	-	±	-	-	-	-	±	±	-
Rifampicin	50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin sulphate	50	-	-	-	±	±	-	-	±	-	±	-	±	-	-	-
	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tetracycline	25	-	-	±	±	±	±	-	±	-	±	±	±	±	±	-
	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

- \- No growth
- ± Very little growth
- \+ Profused growth

Table 10. Antibiotic sensitivity marker for isolates of *R. solanacearum*

Isolates	Antibiotic sensitivity
P1	Amp_{100}^{R} $Carb_{50}^{S}$ Chl_{150}^{S} Gen_{50}^{S} Kan_{50}^{S} Nal_{100}^{S} $Rifam_{100}^{R}$ Str_{50}^{S} Tet_{25}^{S}
P2	Amp_{100}^{R} $Carb_{50}^{S}$ Chl_{150}^{S} Kan_{50}^{S} Nal_{100}^{S} $Rifam_{100}^{R}$ Str_{50}^{S} Tet_{25}^{S}
P3	Amp_{100}^{R} $Carb_{50}^{S}$ Chl_{150}^{S} Gen_{50}^{S} Kan_{50}^{S} Nal_{100}^{S} $Rifam_{100}^{R}$ Str_{50}^{S} Tet_{50}^{S}
P4	Amp_{100}^{R} $Carb_{50}^{S}$ Chl_{150}^{S} Kan_{50}^{S} Nal_{100}^{S} $Rifam_{100}^{R}$ Str_{100}^{S} Tet_{50}^{S}
P5	Amp_{100}^{R} Chl_{150}^{S} Gen_{50}^{S} Kan_{50}^{S} $Rifam_{100}^{R}$ Str_{100}^{S} Tet_{50}^{S}
E1	Amp_{100}^{R} $Carb_{50}^{S}$ Chl_{150}^{S} Gen_{50}^{S} Kan_{50}^{S} Nal_{100}^{S} $Rifam_{100}^{R}$ Str_{50}^{S} Tet_{50}^{S}
E2	Amp_{100}^{R} $Carb_{50}^{S}$ Chl_{150}^{S} Kan_{50}^{S} Nal_{100}^{S} $Rifam_{100}^{R}$ Str_{50}^{S} Tet_{50}^{S}
E3	Amp_{100}^{R} $Carb_{100}^{S}$ Chl_{150}^{S} Kan_{50}^{S} $Rifam_{100}^{R}$ Str_{100}^{S} Tet_{50}^{S}
E4	Amp_{100}^{R} $Carb_{50}^{S}$ Chl_{150}^{S} Gen_{50}^{S} Kan_{50}^{S} Nal_{100}^{S} $Rifam_{100}^{R}$ Str_{50}^{S} Tet_{25}^{S}
E5	Amp_{100}^{R} Chl_{150}^{S} Kan_{50}^{S} Nal_{50}^{S} $Rifam_{100}^{R}$ Str_{100}^{S} Tet_{50}^{S}
W1	Amp_{100}^{R} $Carb_{50}^{S}$ Chl_{150}^{S} Gen_{50}^{S} Kan_{50}^{S} Nal_{50}^{S} $Rifam_{100}^{R}$ Str_{50}^{S} Tet_{50}^{S}
W2	Amp_{100}^{R} $Carb_{50}^{S}$ Chl_{150}^{S} Kan_{50}^{S} Nal_{100}^{S} $Rifam_{100}^{R}$ Str_{100}^{S} Tet_{50}^{S}
W3	Amp_{100}^{R} Chl_{150}^{S} Kan_{50}^{S} $Rifam_{100}^{R}$ Str_{50}^{S} Tet_{50}^{S}
W4	Amp_{100}^{R} $Carb_{100}^{S}$ Chl_{150}^{S} Gen_{50}^{S} Kan_{50}^{S} $Rifam_{100}^{R}$ Str_{50}^{S} Tet_{50}^{S}
W5	Amp_{100}^{R} $Carb_{100}^{S}$ Chl_{150}^{S} Kan_{50}^{S} Nal_{100}^{S} $Rifam_{100}^{R}$ Str_{50}^{S} Tet_{50}^{S}

S- sensitive
R- resistant

4.5.4.1 Plasmid profile

Plasmids from all isolates were extracted by the alkali lysis method and loaded on 0.8 per cent agarose gel. Two bands moving slightly ahead of 21kb fragment of λ *Eco* R I / *Hind* III double digest marker (Plate 6A) were observed in all the isolates.

4.5.4.1.2 Plasmid transformation

E. coli DH 5 α competent cells were transformed with plasmids of three isolates (P3, E3, and W3) of *R. solanacearum.* The banding patterns of plasmid DNA of transformed *E. coli* cells were similar to the bands of native *R. solanacearum* (Plate 6B). The transformed cells were tested for antibiotic sensitivity. These were found resistant to ampicillin and rifampicin (Plate 7), the same antibiotics to which the native bacterial isolates were resistant. This indicated that the genes encoding resistance to these antibiotics could be located on the plasmid.

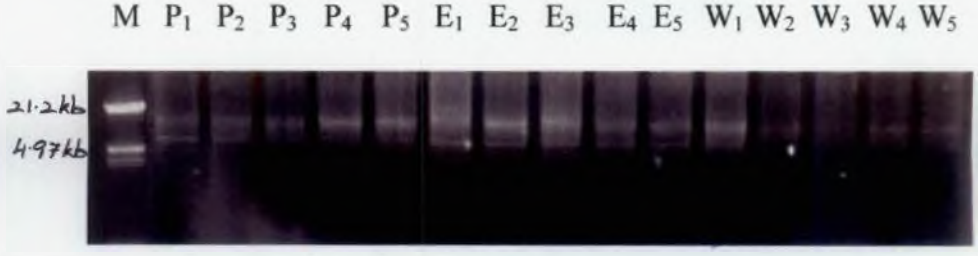
4.5.4.2 RAPD

4.5.4.2.1 Genomic DNA isolation

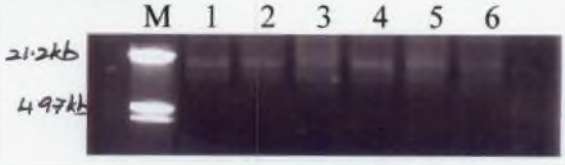
Genomic DNA was extracted from the isolates using large preparation method (Girija, 1999). Agarose gel electrophoresis of genomic DNA preparation revealed the presence of intact bands in all isolates (Plate 8A). The quantity and quality of the isolated DNA were checked using UV visible spectrophotometer. Absorbance at 260 nm and 280 nm was recorded. $OD_{260}nm/OD_{280}nm$ ratio ranged from 1.50 to 2.00. Amount of DNA ranged from 2.00 to 7.50 µg/ml (Table 11).

Plate 6 .

A - Plasmid DNA of *R. solanacearum* isolates

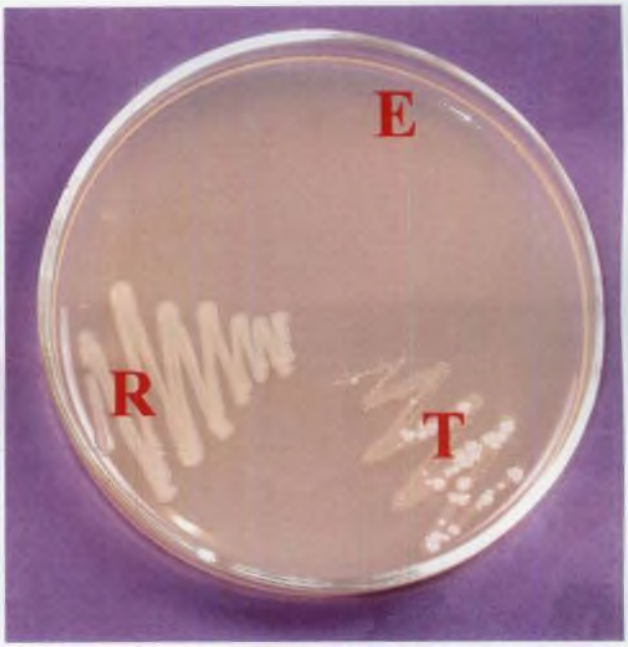


B - Plasmid DNA of *R. solanacearum* and transformed *E. coli* cells



1,3,5 – Plasmid DNA of P3, E3 and W3 isolates
2,4,6 – Plasmid DNA of P3, E3 and W3 transformed *E. coli* cells
M- Molecular weight marker- λ DNA/*Eco* R I *Hind* III

Plate 7.
Growth of transformed *E. coli* cells in presence of ampicillin



E - ***E. coli*** **cells**

R - ***R. solanacearum***

T - Transformed ***E.coli*** **cells**

Table 11. Quality and quantity of DNA extracted from the *R. solanacearum* isolates

Isolates	Optical Density		OD_{260}/OD_{280}	Quantity of DNA (µg/ml)
	260nm	280nm		
P1	0.096	0.050	1.92	4.82
P2	0.120	0.067	1.79	6.00
P3	0.040	0.024	1.66	2.00
P4	0.062	0.033	1.88	3.10
P5	0.053	0.031	1.70	2.65
E1	0.091	0.048	1.89	4.55
E2	0.088	0.047	1.87	4.40
E3	0.090	0.053	1.69	4.50
E4	0.056	0.030	1.86	2.80
E5	0.129	0.071	1.81	6.45
W1	0.056	0.028	2.00	2.80
W2	0.150	0.083	1.80	7.50
W3	0.145	0.076	1.90	7.25
W4	0.117	0.078	1.50	5.85
W5	0.149	0.080	1.86	7.45

Quality of DNA

OD_{260}/OD_{280}	Remarks
1.8-2.0	Good
1.6-1.8, 2.0- 2.2	Fairly Good

4.5.4.2.2 Screening of primers

Initially 20 primers were screened for RAPD with genomic DNA of isolate P1. Sixteen primers giving good amplification were selected and used for further characterization of all the isolates (Plate 8B). Details of selected primers are given in Table 12.

4.5.4.2.3 RAPD Profiles

RAPD profiles revealed variability among the isolates (Plate 8C, 9,10, 11, 12 and 13). The number of bands ranged from 3 to 15. Number of monomorphic bands varied with primer the least having 0 and highest, 7. Percentage polymorphism was calculated for each primer. There was greater variation among the primers in percentage polymorphism, which varied from 100 per cent (recorded by OPU 13, OPU 17 and OPX 9) to 0 per cent (recorded by OPF 8) (Table 13). The banding patterns of OPF 8 profile were similar with the race 3 marker of James (2001). All the isolates produced similar bands with size 1.45 kb.

4.5.2.4 Analysis of RAPD profiles

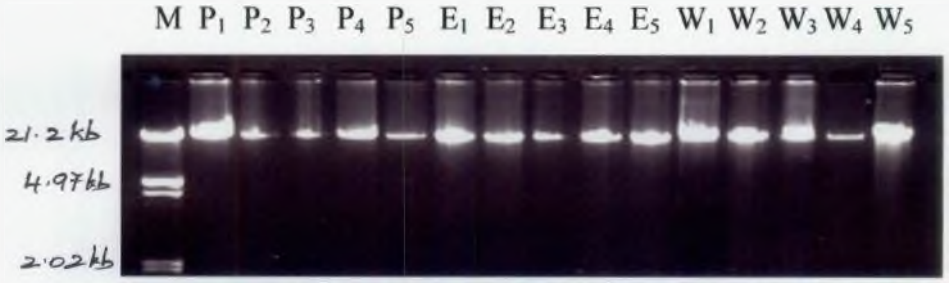
Genetic similarity was computed from RAPD profiles as Jaccards coefficient using NTSYS PC.2.0 software (Table 14). Dendogram was constructed by using UPGMA routine (Fig 1). Highest similarity value was 0.76 and it was observed between P1 and P5, followed by 0.70 between E2 and E4. Lowest similarity value was 0.49 observed between P1 and W1, followed by 0.50 between W1 and W4. The dendrogram consisted of one major cluster and one small sub cluster. The major cluster consisted of all isolates except E1 and W1, which formed a separate sub

Table 12. Details of primers used for characterization of *R. solanacearum* isolates through RAPD

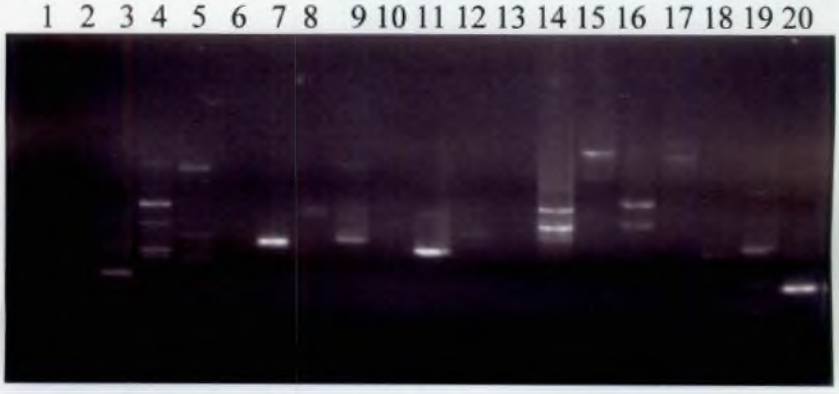
Sl. No	Primer code	Primer sequence
1	OPF 8	GGGATATCGG
2	OPU 6	ACCTTTGCGG
3	OPU 7	CCTGCTCATC
4	OPU 8	GGCGAAGGTT
5	OPU 9	CCACATCGGT
6	OPU 10	ACCTCGGCAC
7	OPU 11	AGACCCAGAG
8	OPU 13	GGCTGGTTCC
9	OPU 15	ACGGGCCAGT
10	OPU 17	ACCTGGGGAG
11	OPU 18	GAGGTCCACA
12	OPU 19	GTCAGTGCGG
13	OPU 20	ACAGCCCCCA
14	OPX 3	TGGCGCAGTG
15	OPX 4	CCGCTACCGA
16	OPX 5	CCTTTCCCTC
17	OPX 6	ACGCCAGAGG
18	OPX 7	GAGCGAGGCT
19	OPX 8	CAGGGGTGGA
20	OPX 9	GGTCTGGTTG

Plate 8 .

A - Genomic DNA of *R. solanacearum* isolates



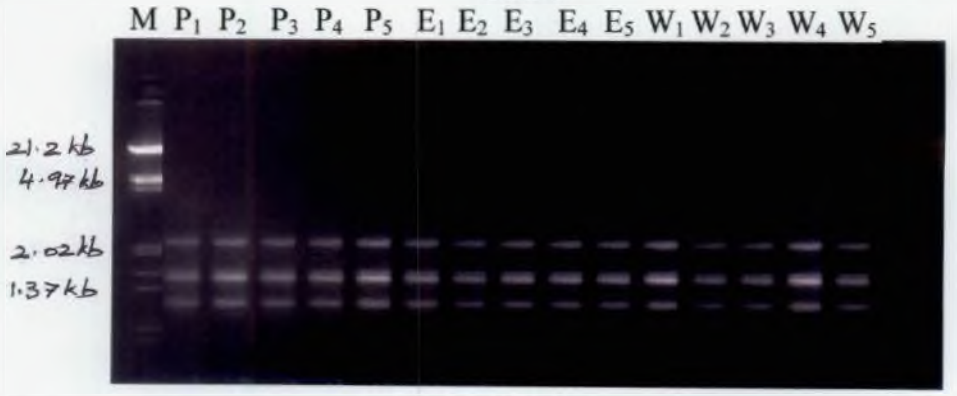
B - Primer screening for RAPD analysis



1- OPU 8	2-OPU 6	3-OPU 9	4-OPU 11	5-OPU 13
6-OPU 10	7-OPU 15	8-OPU 7	9-OPU 17	10-OPU 18
11-OPU 20	12-OPU 19	13-OPX 3	14-OPF 8	15- OPX 4
16-OPX 9	17-OPX 7	18-OPX 8	19-OPX 6	20-OPX 5

C - RAPD profiles of *R. solanacearum*

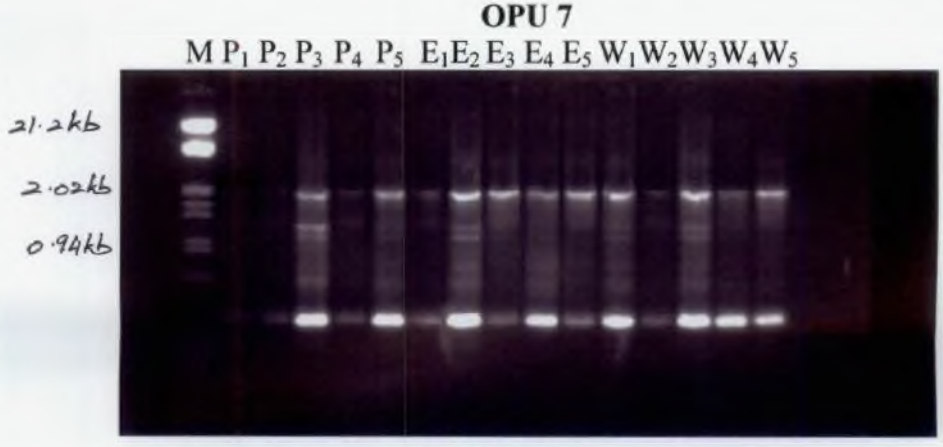
OPF 8



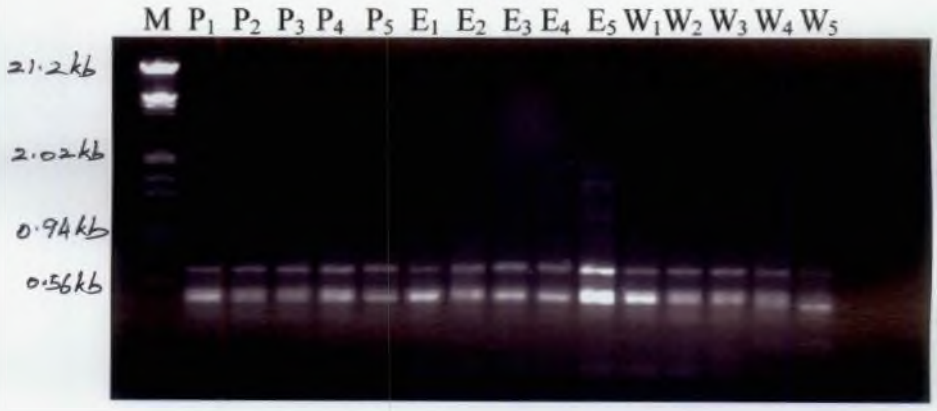
M- Molecular weight marker- λ DNA/*Eco* R I *Hind* III

Plate 9 .
RAPD profiles of *R. solanacearum*

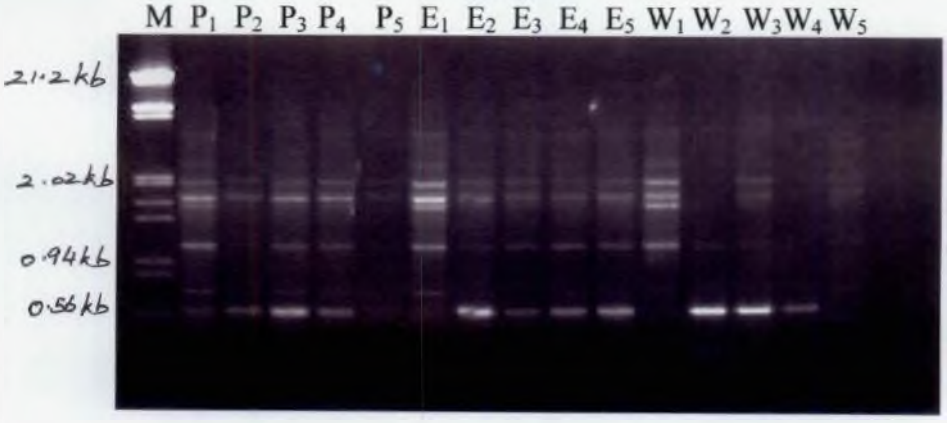
OPU 7



OPU 8



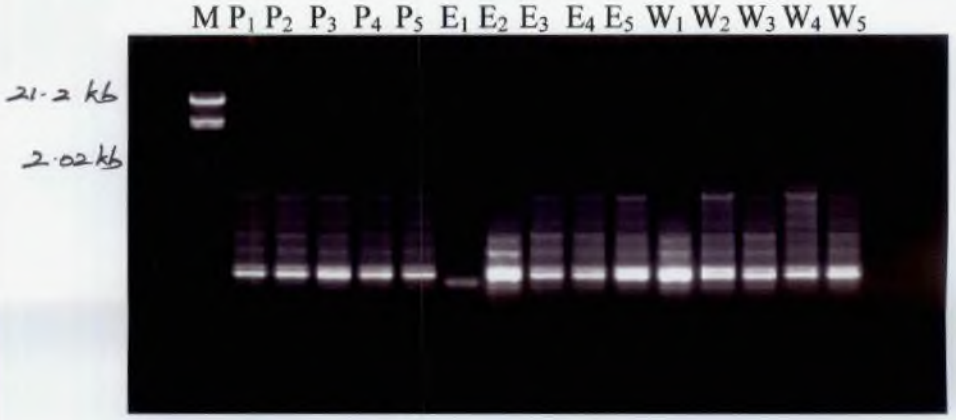
OPU 9



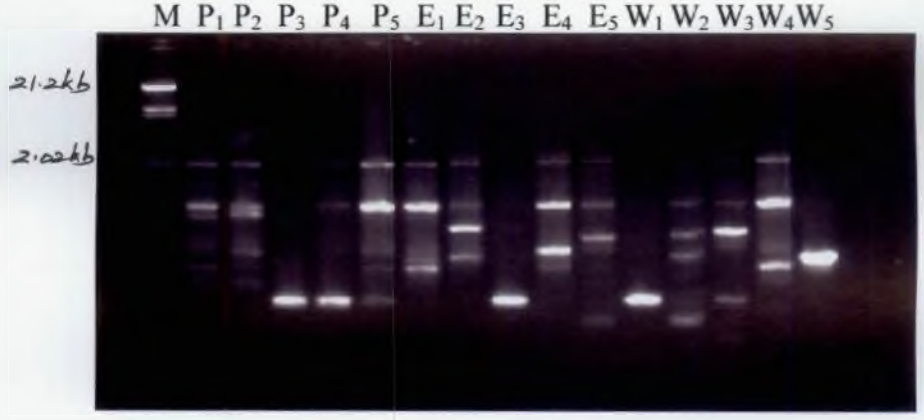
M- Molecular weight marker- λ DNA/*Eco*R I *Hind* III

Plate 10 .
RAPD profiles of *R. solanacearum*

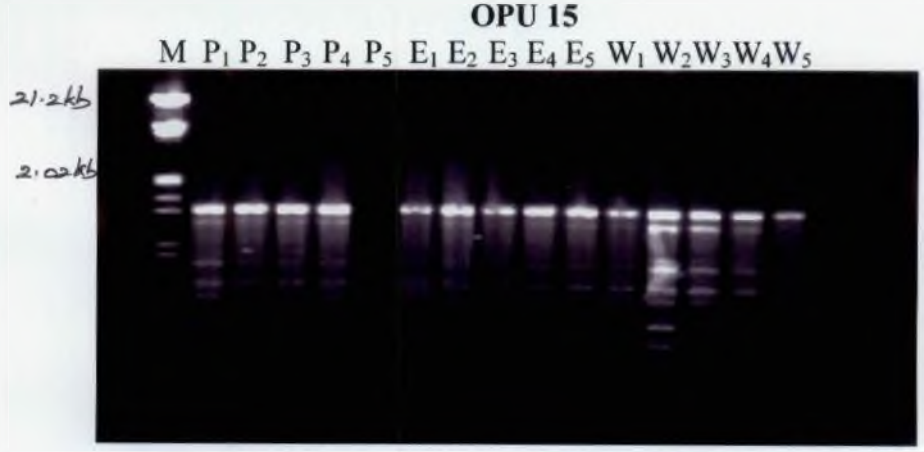
OPU 11



OPU 13



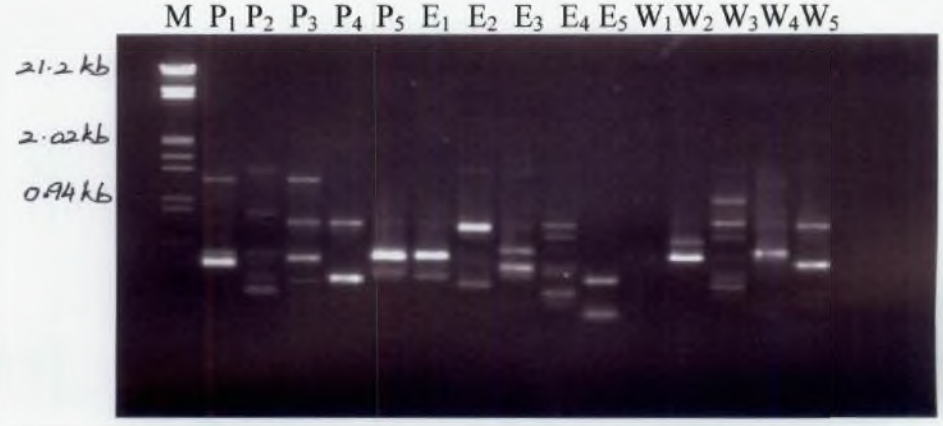
OPU 15



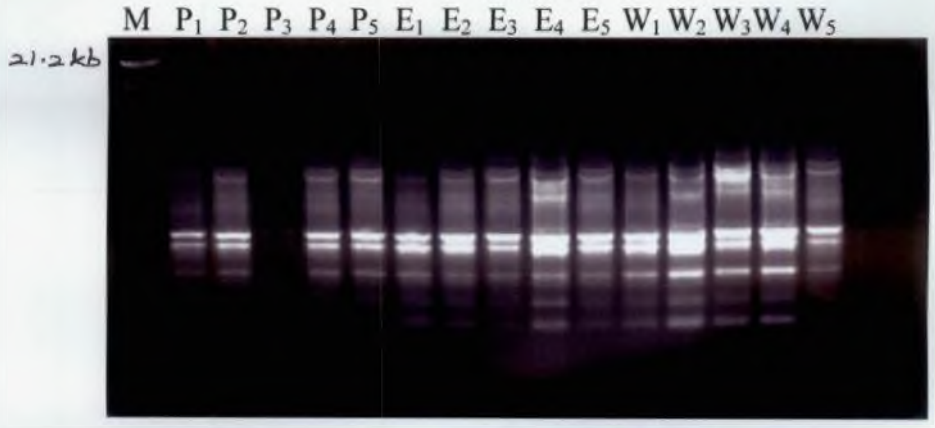
M- Molecular weight marker- λ DNA/*Eco* R I *Hind* III

Plate 11 .
RAPD profiles of *R. solanacearum*

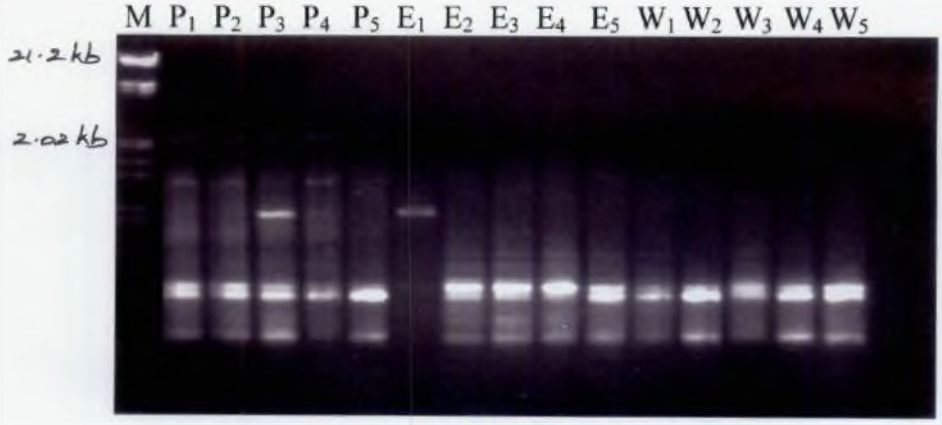
OPU 17



OPU 19



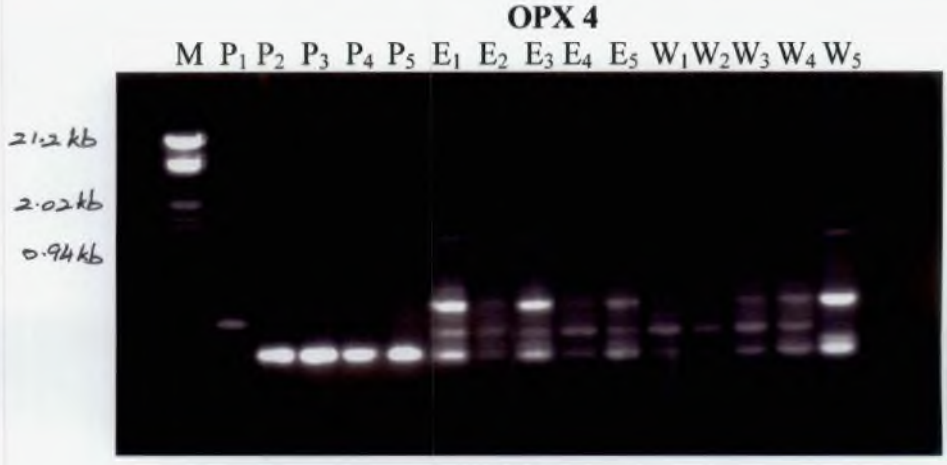
OPU 20



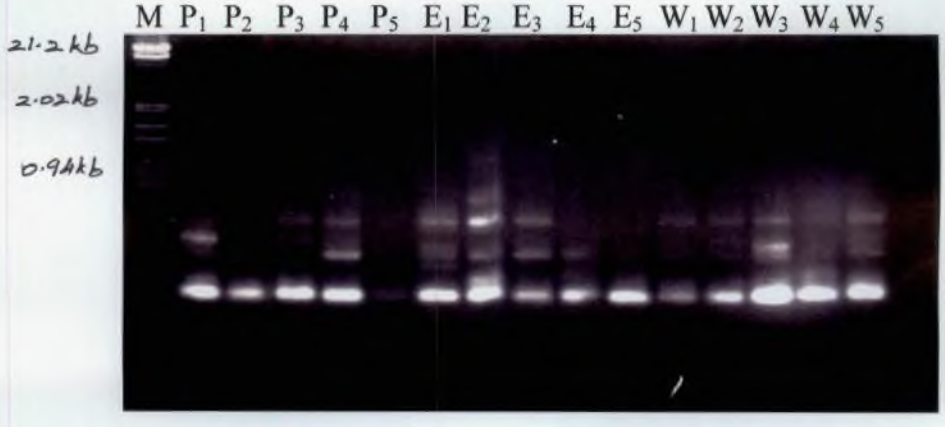
M- Molecular weight marker- λ DNA/*Eco* R I *Hind* III

Plate 12 .
RAPD profiles of *R. solanacearum*

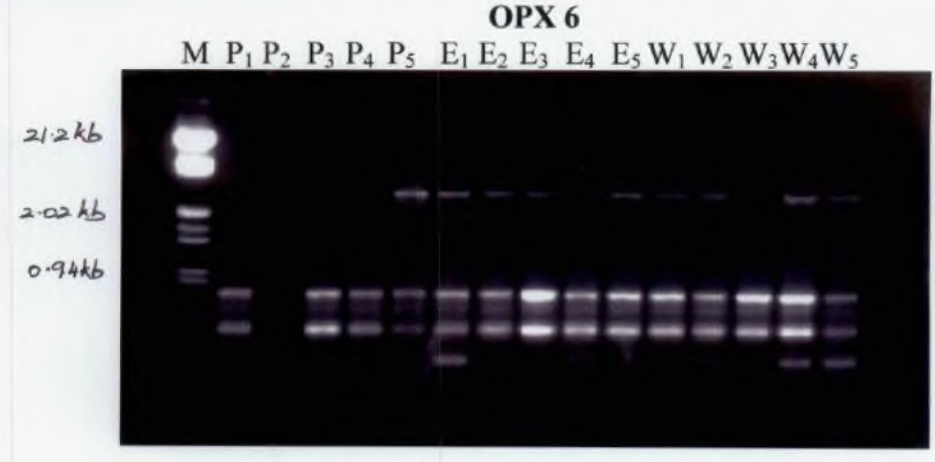
OPX 4



OPX 5



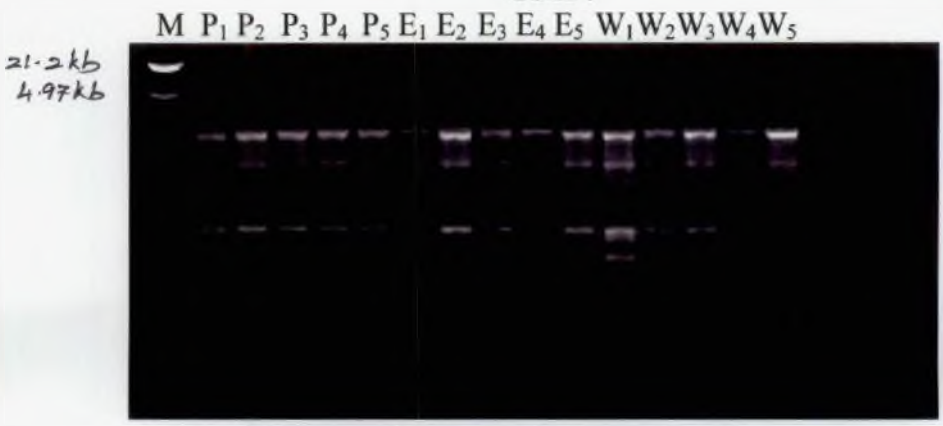
OPX 6



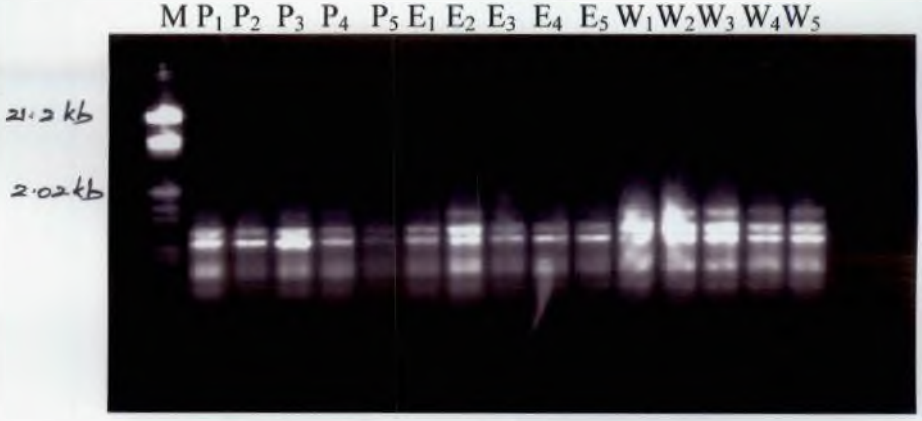
M- Molecular weight marker- λ DNA/*Eco* R I *Hind* III

Plate 13 .
RAPD profiles of *R. solanacearum*

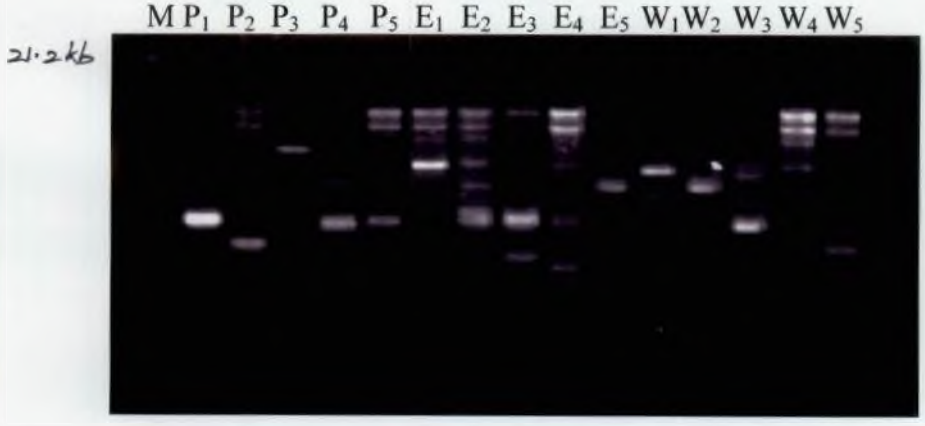
OPX 7



OPX 8



OPX 9



M- Molecular weight marker- λ DNA/*Eco* R I *Hind* III

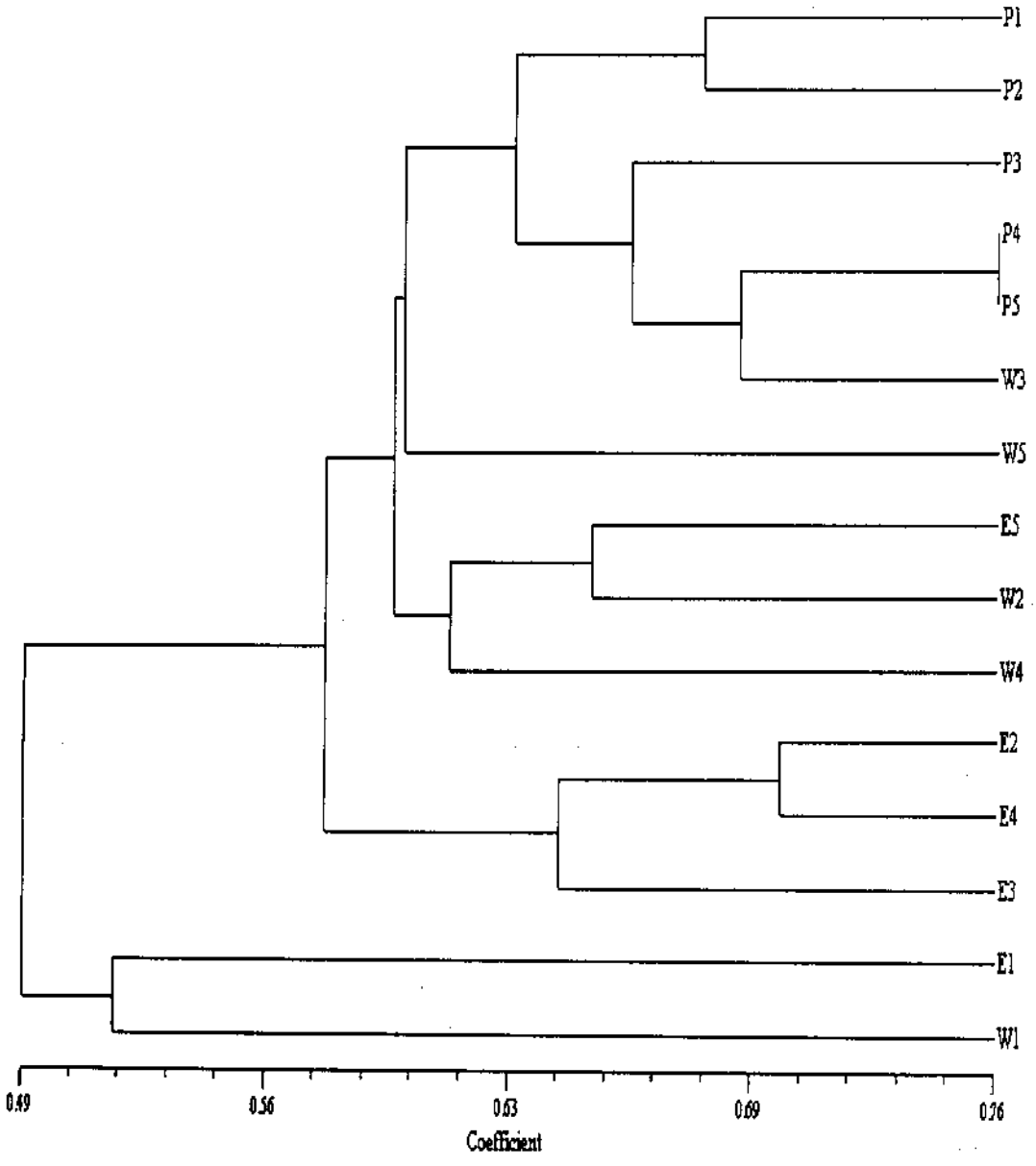
Table 13. Per cent polymorphism for different primers

Primers	Total no of bands	No of polymorphic bands	No of monomorphic bands	Per cent polymorphism
OPF 8	3	0	3	0.0
OPU 7	6	4	2	66.6
OPU 8	6	1	5	16.6
OPU 9	8	7	1	87.5
OPU 11	7	3	4	42.8
OPU 13	11	11	0	100.0
OPU 15	7	3	4	42.8
OPU 17	15	15	0	100.0
OPU 19	10	7	3	70.0
OPU 20	10	3	7	30.0
OPX 4	5	1	4	20.0
OPX 5	6	3	3	50.0
OPX 6	5	3	2	60.0
OPX 7	7	6	1	85.7
OPX 8	5	1	4	20.0
OPX 9	11	11	0	100.0

Table 14. Genetic similarity matrix of RAPD profiles

	P1	P2	P3	P4	P5	E1	E2	E3	E4	E5	W1	W2	W3	W4	W5
P1	1														
P2	0.68	1													
P3	0.62	0.60	1												
P4	0.63	0.68	0.68	1											
P5	0.65	0.68	0.68	0.76	1										
E1	0.53	0.52	0.54	0.53	0.56	1									
E2	0.55	0.53	0.57	0.60	0.62	0.54	1								
E3	0.51	0.56	0.59	0.58	0.62	0.53	0.63	1							
E4	0.54	0.56	0.54	0.58	0.66	0.55	0.70	0.65	1						
E5	0.58	0.64	0.58	0.64	0.62	0.56	0.59	0.59	0.59	1					
W1	0.54	0.49	0.57	0.52	0.51	0.54	0.54	0.52	0.53	0.51	1				
W2	0.56	0.54	0.54	0.55	0.59	0.58	0.59	0.51	0.54	0.65	0.51	1			
W3	0.56	0.60	0.62	0.67	0.71	0.57	0.67	0.61	0.60	0.64	0.57	0.62	1		
W4	0.59	0.63	0.54	0.55	0.63	0.54	0.60	0.51	0.60	0.58	0.50	0.64	0.63	1	
W5	0.54	0.58	0.60	0.59	0.65	0.56	0.55	0.59	0.54	0.58	0.51	0.60	0.62	0.57	1

Fig. 1 Dendrogram obtained from the pooled data of RAPD profiles



cluster. Within the major cluster all the Palakkad isolates formed a separate sub cluster indicating less diversity among them.

Discussion

5. DISCUSSION

Ginger is one of the oldest and renowned commercial spices esteemed for its aroma, flavour and pungency. It is much valued as a spice, medicine as well as vegetable since very ancient days. India is the largest producer and Kerala occupies the highest share in total production. Though the state ranks first in production, bacterial wilt caused by *R. solanacearum* is one of the major constraints in ginger cultivation. Earlier, the pathogen was characterized based on cultural, morphological and biochemical tests. Since conventional method of characterization takes long duration and it involves repeated culturing and subjecting to various biochemical tests the present study was done using molecular techniques. Recently, these techniques are gaining more importance as they are highly specific, rapid and can even detect small variations among closely related strains.

In Kerala, ginger cultivation is mainly confined to three districts namely Palakkad, Ernakulam and Wynad. A total of 15 samples, five from each districts were used for the study. Ginger plants showing typical bacterial wilt symptoms were collected from farmers fields and subjected to ooze test to confirm the bacterial infection. Flow of bacterial ooze was observed from the cut ends immersed in water. Similar findings were also obtained by Mathew *et al.* (2002) and Tsuchiya *et al.* (2002) while studying *R. solanacearum* strains infecting ginger.

The bacteria were isolated on triphenyl tetrazolium chloride (TZC) medium and their cultural and morphological characters were studied. TZC is a basic component of various selective media developed by earlier workers. The bacterium appeared as small creamy white colonies with pink centre. Apart from better recovery rate, TZC medium was also efficient enough to differentiate wild and variant types. In the present study, all isolates of *R. solanacearum* produced small creamy white colonies

with pink centre. The same media was used by the earlier workers for the isolation of the bacterium from various host plants (Husain and Kelman, 1958; Khan *et al.*, 1979; Nayar, 1982; He *et al.*, 1983; Swanepoel and Young, 1988; Prior and Steva, 1990; Rani, 1994; Paul, 1998; Mathew *et al.*, 2000 and James, 2001).

Maintenance of virulence of *R. solanacearum* in culture is reported to be difficult. Earlier workers maintained the pathogen on PDA slants covered with sterile mineral oil (Kelman, 1954). The culture was found virulent for a long period of 6½ years. Later, a convenient method of storing as stock culture was developed (Husain and Kelman, 1958). Here, five loopful of typical colonies were suspended in sterile water and stored at 25°C. This method was also followed by several workers (Kelman and Person, 1961; Khan *et al.*, 1979; Granada and Sequeira, 1983 and Swanepoel and Young, 1988). Several other workers found that stock cultures can also be maintained at room temperature (He *et al.*, 1983; Prior and Steva, 1990 and Mathew *et al.*, 2000). The cultures were found free from avirulent mutant for about 18-24 months. In contrary to this, James (2001) observed mutant types with non-mucoid colonies, when pure cultures of 5-6 months were streaked on TZC medium. However, in this study, the stock cultures were stored at 4°C and tested at regular intervals of one month upto 18 months, yielded typical creamy white colonies with pink centre and were free from avirulent mutant colonies. Similar method of preservation was also adopted by Kumar *et al.* (1993)

Among the different inoculation methods adopted for establishing pathogenicity, pseudostem inoculation yielded cent per cent disease incidence and early appearance of symptoms, showing that this is the best method for the inoculation of the pathogen causing bacterial wilt in ginger. However, James (2001) and Mathew (2001) observed leaf clipping as the best method of inoculation for *R. solanacearum* infecting solanaceous vegetables.

R. solanacearum is reported to be a complex species, existing as different biovars and races. Strains of *R. solanacearum* were earlier classified into races based on host range (Buddenhagen *et al.*, 1962). Later, Lozano and Sequeira (1970) grouped races based on hypersensitivity reaction on tobacco. In this study, race classification of *R. solanacearum* infecting ginger was done based on hypersensitivity reaction on capsicum and this was confirmed by molecular characterization. Hypersensitivity is the process in which rapid death of cells around the point of infection of the pathogen occurs. It is the sign of high resistance approaching immunity. The leaves of the capsicum plants infiltrated with bacterial ooze remained symptomless for 24-48 h, water soaked lesions developed after 72 h. By 96 h yellowing was noticed around the lesion, which spread throughout the entire leaf causing defoliation. The above criteria were in accordance with the race 3 descriptions of Lozano and Sequeira (1970).

In addition to this, an attempt using molecular technique also confirmed the grouping of the pathogen under race 3. James (2001) identified a race 3 isolate of *R. solanacearum* infecting solanaceous vegetables from Ambalavayal region of Kerala based on RAPD technique. A 1.45 kb band was obtained for all isolates of race 3 in RAPD with OPF 8 as the primer. Persley *et al.* (1985) classified races based on host range, geographical distribution and ability to survive under different environmental conditions and classified ginger strain under the group race 4. A search on literature revealed no information regarding the grouping of ginger strain of *R. solanacearum* except the report of Persely *et al.* (1985). Hence this is the first report regarding the identification of race 3 isolate of *R. solanacearum* infecting ginger from any part of the world.

The ability of any pathogen to infect plants other than its own host favours its long term survival. In the present study, the ginger isolates, upon cross inoculation were found to infect only tomato and brinjal but not chilli and other cucurbitaceous vegetables like snake gourd, pumpkin, ash gourd and bitter gourd, indicating that only tomato and brinjal are the other collateral hosts of *R. solanacearum* causing bacterial wilt in ginger plants. The ability of ginger isolates to produce wilt on tomato was also reported by earlier workers (Ishii and Aragaki, 1963 and He *et al.*, 1983). However, the ability of ginger isolates to produce wilting on brinjal was in contrary to the earlier findings (Zehr, 1969; Nayar, 1982; Jyothi, 1992 and Rani, 1994). On cross inoculation of *R. solanacearum* isolates from tomato, brinjal and chilli on ginger plants, only the isolate from chilli was found to cause wilt symptom. These results are in confirmation with the results obtained by Jyothi (1992). The inability of tomato and brinjal isolates to cause wilting in ginger was supporting the earlier work of Jyothi (1992) and Rani (1994).

All the isolates produced creamy white colonies after 24 h and later by 48 h a light pink colour developed at the centre. The colonies were circular with fluidal mass. Colony characters were also related with the pathogenicity (Kelman, 1954). Highly pathogenic or virulent strains were found to produce fluidal white colonies with pink centre while weakly pathogenic or avirulent strains were found to produce butyrous red colonies. Based on the above characters, the isolates were found to belong to wild or virulent types. Similar colony types were also obtained by other workers (Mathew *et al.*, 1979; He *et al.*, 1983; Prior and Steva, 1990; Jyothi, 1992; Rani, 1994; Mathew *et al.*, 2000 and James, 2001).

All isolates were basically similar in terms of biochemical characteristics. The isolates formed viscous thread like structure with KOH, indicating Gram negative reaction. This was found to be the effective method to identify Gram reaction

(Suslow *et al.*,1982). Gram staining was also done to confirm this reaction. All the isolates were found to possess oxidase enzyme. However, 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). This test was found be important as it distinguishes *R. solanacearum* from other members of Pseudomonads. Mostly all isolates took around one minute to develop purple colour. In case of catalase test, all the isolates were found to produce air bubbles. This indicates the conversion of hydrogen peroxide (a by product of aerobic respiratory metabolism) to water and oxygen by catalase enzyme of the pathogen. The isolates were negative for arginine dihydrolase reaction indicating the inability to produce ammonia from arginine under anaerobic condition. This result were in confirmation with the results reported by earlier workers (Devi, 1978; Mathew *et al.*, 1979; Jyothi, 1992; Rani, 1994; Mathew *et al.*, 2000; James, 2001 and Mathew *et al.*, 2002). In denitrification test, all the isolates showed positive reaction by producing gas from the nitrate source. Glucose was oxidised by all isolates as indicated by colour change of the medium to yellow and presence of air bubbles. The present findings are in agreement with the results reported by Jyothi (1992) and Rani (1994).

Sugar utilization by the pathogen was the criteria taken for the grouping of isolates into biovars. All the isolates utilized all sugars except dulcitol indicating a variation in the group biovar III. It was considered as a sub type of biovar III and designated as biovar III A, which was supported by the findings of Mathew *et al.* (2002). Even though slight variation was noted among the isolates with respect to sugar utilization time, this was not taken into consideration for the grouping of the pathogen. In India, Kumar *et al.* (1993) made the first report of biovar III A infecting brinjal, tomato and capsicum in Himachal Pradesh. In Kerala, James (2001) found biovar III A infecting chilli and tomato and Mathew (2001) also reported biovar III A infecting chilli, brinjal and tomato. However, the earlier report by Rani (1994)

indicated that ginger isolate belonged to biovar III, whereas ginger isolates from Japan and China were grouped under biovar IV (Poussier *et al.*, 2000 and Tsuchiya *et al.*, 2002).

Use of antibiotics is considered to be the most effective method for controlling any bacterial disease. Hence an attempt was made to screen the isolates for antibiotics sensitivity. All the isolates were found to be resistant to ampicillin and rifampicin upto a concentration of 100 µg/ml. They were highly sensitive to chloramphenicol at 150 µg/ml and kanamycin at 50 µg/ml. Similar result was obtained by James (2001) while screening isolates of solanaceous vegetables. On the contrary, *R. solanacearum* isolates were found to be sensitive to ampicillin and resistant to chloramphenicol by other workers (He *et al.*, 1983 and Prior and Steva, 1990). Intrinsic antibiotic resistance pattern is expected to vary greatly with different isolates, especially if these traits are located on plasmid DNA.

To take the advantage of the efficiency of molecular techniques in detecting even small variations among closely related strains, attempts were made to characterize ginger isolates using plasmid profile and RAPD technique.

Plasmid DNA isolated using alkali lysis method (Birnboim and Doly, 1979) showed presence of two distinct bands of approximately 21 kb size indicating the possibility of existence of two plasmids. However, restriction analysis has to be done to confirm whether these bands are two different forms of the same plasmid or two different plasmids. Similar findings were also reported by earlier workers (Morales and Sequeira, 1985). In order to find the location of antibiotic resistance genes within the bacteria either on genomic DNA or plasmid, plasmids were transformed to *E. coli* DH 5 α competent cells. The transformed cells were tested for resistance to the antibiotics to which the native bacteria showed resistance. Transformants were

resistant to ampicillin and rifampicin and it confirms the possibility of location of antibiotic resistance genes on plasmids.

Genomic DNA was extracted from all the isolates. The quality of DNA was good as revealed by gel electrophoresis and the quantity ranged from 2.0 to 7.5 µg/ml (Plate 8A and Table 11).

For RAPD analysis 20 random primers available at CPBMB were screened using the genomic DNA of P1 isolate. Sixteen primers gave good amplification and were used for further characterization of all isolates. Number of bands obtained for each primer ranged from 3 to 15. Percent polymorphism ranged from 0 to 100 per cent. The primers OPU 13, OPU 17 and OPX 9 gave 100 per cent polymorphism indicating the presence of heterogenous sequences at some regions of the genomic DNA. Amplification by other primers has given little polymorphism indicating the existence of homogenous sequences. However, contrary to the above findings Kumar and Sarma (2000), while characterizing ginger strains using RAPD obtained monomorphic bands indicating the presence of single strain.

Genetic similarity values were computed from RAPD profiles using Jaccards coefficient. Highest similarity value was obtained between P4 and P5 isolates. Dendrogram obtained through cluster analysis showed two clusters one major and one sub cluster. The major cluster consisted of all isolates except E1 and W1, which formed the small sub cluster. Within the major cluster all the Palakkad (P1, P2, P3, P4 and P5) isolates formed a common sub cluster indicating more similarity among them. The ginger growers of these regions were getting their seed material from a single location (Adiperandu village of Ayallur panchayat). Farmers of Wynad region was procuring their seed materials from various locations of Pulpally, Kalpetta and Kodugu areas. Ginger growers of Ernakulam districts were using the local seed

materials. Infected seed material was found to be the major inoculum source for the development and spread of the wilt disease (Kumar and Sarma, 2000).

From the above results it is evident that molecular analysis using RAPD markers showed much diversity among the isolates even though they showed similar response in cultural and biochemical tests. Therefore, it can be used as an effective tool to detect even small variations among *R. solanacearum* strains. Similar findings of molecular characterization revealing more diversity than by other methods was also reported by Darasse *et al.* (1997). Further work on molecular characterization has to be done using more primers in order to make a complete coverage of the whole genomic sequences of *R. solanacearum*.

Summary

6. SUMMARY

An investigation was undertaken at Department of Plant Pathology and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, to characterize *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* causing bacterial wilt in ginger using molecular marker during January 2002-August 2003. In this study *R. solanacearum* strains infecting ginger, collected from ginger growing tracts of Palakkad, Ernakulam and Wynad were characterized at cultural, biochemical and molecular levels. The salient results of the study were

1) The pathogenic bacteria from the diseased plant samples of these tracts were isolated on TZC medium.

2) The pathogen maintained as stock cultures in sterile water at 4 °C retained virulence upto 18 months and did not give rise to avirulent mutants.

3) Pathogenicity of the isolates was proved using pseudostem inoculation method, as this was found to be more effective than other methods. Typical wilting symptoms (yellowing of leaves along the margin, inward rolling and easy detachment of pseudostem) were produced in ginger within a period of six days.

4) The isolates were grouped into races based on hypersensitive reaction on capsicum. All the isolates were found to belong to race 3. This was further confirmed by DNA amplification using OPF 8 primer.

5) In host range studies, the ginger strains were found to infect only tomato and brinjal. Cross inoculation of chilli isolate on ginger produced disease symptoms, but other isolates from tomato and brinjal were unable to infect it.

Chilli and cucurbits were found to be non-host of *R. solanacearum* infecting ginger. On cross inoculation of solanaceous isolates of *R. solanacearum* on ginger, only chilli isolate was found to infect ginger.

6) All the isolates produced similar colonies on TZC medium. Colonies were small, creamy white with pink centre and surrounded with fluidal mass.

7) The isolates showed positive reaction for solubility in KOH, nitrate reduction, production of catalase and oxidase enzymes, and fermentation of glucose. None of the isolates produced ammonia from arginine.

8) All the isolates oxidised lactose, maltose, cellobiose, mannitol and sorbitol but failed to oxidise dulcitol. Based on this reaction, they were classified under biovar III A.

9) The isolates were found resistant to ampicillin and rifampicin, but they were sensitive to chloramphenicol and kanamycin. However, for other antibiotics they showed varied response.

10) Plasmid profiles of all isolates showed two distinct bands of approximately 21 kb size indicating the possibility of presence of two plasmids.

11) The plasmids, when transformed to *E. coli* DH 5 α competent cells, imparted resistance to both ampicillin and rifampicin, the antibiotics to which native *R. solanacearum* isolates were resistant. This indicated the possibility of location of antibiotics resistant genes on plasmids.

12) Genomic DNA extracted from all isolates were of good quality and quantity. The quantity ranged from 2 to 7.5 µg/ml.

13) RAPD analysis using 16 primers revealed polymorphism among the isolates. Primers OPU 13, OPU 17 and OPX 9 yielded 100 per cent polymorphism.

14) Dendrogram constructed using the data showed two clusters, one major and one sub cluster. Palakkad isolates formed a single cluster indicating similarity among themselves.

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* Originals not seen

Appendix

APPENDIX

A. Composition of media used in the study.

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 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

3. 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

4. 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

5. Tryptone Yeast Extract (TY) medium

Tryptone	-	10.0 g
Yeast Extract	-	5.0 g
Bacto Agar	-	20.0 g
Distilled water	-	1 l

6. Luria Bertani (LB) medium

Tryptone	-	10.0 g
Yeast Extract	-	5.0 g

NaCl	-	5.0 g
Glucose	-	1.0 g
Distilled water	-	1 l
pH	-	7

7. Yeast Extract Tryptone (2YT) medium

Tryptone	-	16 g
Yeast extract	-	5 g
NaCl	-	5 g
Distilled water	-	1 l
pH	-	7.0

B. Buffers and solutions

1. Resuspension buffer (for plasmid isolation)

50 mM Tris Cl pH 8.0
10 mM EDTA
0.2 mg/ml RNase A

2. Lysis buffer (for plasmid DNA isolation)

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.

CHARACTERIZATION OF *Ralstonia solanacearum* (Smith) YABUUCHI *et al.* CAUSING BACTERIAL WILT IN GINGER USING MOLECULAR MARKER

By

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ABSTRACT OF THE THESIS

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ABSTRACT

In Kerala, bacterial wilt caused by *Ralstonia solanacearum* is one of the major constraints in ginger cultivation. Earlier, the pathogen was characterized based on cultural, morphological and biochemical tests. Since these methods are time consuming and involve repeated subculturing and tedious biochemical tests, an attempt was made to characterize the pathogen at molecular level using plasmid profile and RAPD technique.

Wilted plant samples were collected from ginger growing tracts of Palakkad, Eranukulam and Wynad districts. The pathogen was isolated on TZC medium, it produced creamy white pink centred fluidal colonies. Stock cultures prepared by suspending single colonies in sterile water were maintained at 4 °C.

Pathogenicity of the isolates was established using pseudostem inoculation method. Tomato and brinjal were found to be collateral hosts of the pathogen. Based on hypersensitivity reaction on capsicum the isolates were identified as race 3. This was further confirmed by RAPD assay.

The isolates were found to be Gram negative and showed positive reaction for solubility in KOH, nitrate reduction, production of catalase and oxidase enzyme, fermentation of glucose. All the isolates utilized lactose, maltose, cellobiose, manitol, sorbitol but not dulcitol and hence, were grouped as biovar III A.

Plasmid profile developed for the isolates showed presence of two bands of approximately 21 kb size. Plasmids when transformed to *E. coli* DH 5α cells, conferred resistance to ampicillin and rifampicin, indicating that the genes encoding resistance to these antibiotics were located on the plasmid.

RAPD analysis using 16 primers showed much diversity among the isolates. Primers OPU 13, OPU 17 and OPX 9 showed 100 per cent polymorphism. Dendrogram obtained through cluster analysis showed one major and one sub cluster. All the Palakkad isolates were grouped under a single cluster.

The present study indicated possibility of using molecular marker as a tool to detect even slight variability among *R. solanacearum* isolates infecting ginger.