CHARACTERISATION OF *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* INFECTING SOLANACEOUS VEGETABLES IN RELATION TO PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF SOIL

By

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(2017-11-015)

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2019

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THESIS

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DEPARTMENT OF PLANT PATHOLOGY
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2019

DECLARATION

I, Anjali V.A. (2017-11-015) hereby declare that this thesis entitled **"Characterisation of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* infecting solanaceous vegetables in relation to physico-chemical and biological properties of soil"** is a bonafide record of research work done by me during the course of research and that the thesis has not been previously formed for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"Characterisation of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* infecting solanaceous vegetables in relation to physico-chemical and biological properties of soil"** is a record of research work done independently by **Ms. Anjali V.A.** (2017-11-015) under my guidance and supervision and that it has not been previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

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1. INTRODUCTION

India is bestowed with a wide range of climatic and geographical conditions which makes it suitable for growing various kinds of horticultural crops such as fruits, vegetables, flowers, nuts, spices and plantation crops. Among these, vegetables occupy a major place as they are a rich source of essential minerals, vitamins and fiber required for maintaining human health. India stands second in vegetable production in the world with an annual production of 168 million tonnes (GOI, 2018). But the vegetable production in Kerala is much less compared to other states with an annual production of only 1.64 million tonnes (GOI, 2018). The incidence of diseases and pests is one of the major constraints in vegetable production, mainly attributed to the warm humid climate prevailing in the state.

Among the diseases, bacterial wilt of solanaceous crops stands as a major limiting factor affecting the vegetable production in Kerala. The causal agent, *Ralstonia solanacearum* (Smith) Yabuuchi *et al*., has been ranked as the second most important bacterial pathogen in the world next to *Pseudomonas syringae*. The disease is highly destructive as the symptoms include rapid and fatal wilting of host plants. The existing soil-plant atmosphere continuum in Kerala is extremely favourable for bacterial wilt (Mathew and Peter, 2004) and it can result in 100 per cent yield loss in susceptible varieties under favorable weather conditions. Bacterial wilt has been reported from different geographical terrains infecting a wide range of crops in Kerala. The wide host range and geographical distribution of the pathogen coupled with high variability makes its management extremely challenging.

R. solanacearum exhibits high diversity, demonstrating variation in host range, geographical distribution, pathogenicity, epidemiological relationship and physiological properties. Owing to the very high degree of variability, it is considered as a species complex. Biovar typing and race assessment are the methods commonly used for assessing the diversity of the bacterium and classification of different strains. Many molecular characterisation techniques are also being used to establish the variability of the pathogen. The complexity and diversity has necessitated classification of the species into phylotypes and sequevars based on sequence homology of the genetic material.

Such diversity exhibited by the pathogen hampers the breeding of resistant cultivars, consequently the resistant cultivars produced in one geographic area may not be resistant to bacterial strains present in another area. This situation necessitates the characterisation of the bacterial pathogen from different agro ecological units of Kerala, which will help in developing a better understanding of the pathogen and hence design the breeding and management programmes accordingly.

Generally, plant diseases caused by soil borne pathogens such as *R. solanacearum* result from a set of multiple and complex interactions, including both biotic and abiotic factors prevailing in the soil. Abiotic factors such as nutrient (organic matter and minerals) conditions, soil type, pH, anaerobic conditions, temperature, and moisture content influence the multiplication of *R. solanacearum* in soil. Biotic factors are mainly attributed to the density and diversity of soil microflora and degree of susceptibility of the crops grown. Hence an understanding of the soil physico-chemical and biological properties influencing the pathogen as well as bacterial wilt incidence could serve as a tool in adopting appropriate preventive and management measures against the disease. Considering the importance of bacterial wilt, affecting solanaceous crops in Kerala, the present investigation was carried out with an objective to isolate and characterise *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* infecting solanaceous vegetables from four different agro ecological units of Kerala and to study the soil physico-chemical and biological properties influencing the pathogen. The study comprised of the following experiments,

- Purposive sampling survey to collect bacterial wilt infected plant samples as well as rhizosphere soil samples of diseased and healthy plants from four different agro ecological units of Kerala
- Isolation and characterisation of the *Ralstonia solanacearum* isolates from different locations
- Analysis of physicochemical properties of rhizosphere soil samples of healthy and diseased plants
- Enumeration of rhizosphere microflora of diseased and healthy plants

2. Review of literature

2. REVIEW OF LITERATURE

The wilt disease of solanaceous crops caused by the bacterial pathogen *Ralstonia solanacearum* is a major devastating soil borne disease in the world, limiting the production of solanaceous vegetable crops, and affecting a wide range of plant species. The loss due to this disease is very high, ranging from two to 100 per cent depending on environmental conditions and crops. Bacterial wilt affects crops of economic importance in almost all the tropical, subtropical and warm temperate regions of the world.

2.1 Disease occurrence and distribution

The first report of bacterial wilt was from Italy in 1882 (Walker, 1952). Years later, Burill (1890) studied the disease in association with rot of Irish potato tubers in United States. However, Smith (1896) was the first to describe the disease and causal agent as *Bacillus solanacearum.* The disease also appeared in South Africa in 1914 (Doidge, 1914) and in Pakistan in 1989 (Geddes, 1989). Hayward (1991) reported that bacterium is especially destructive in moist soils and has an unusually broad host range including 450 host species in 54 botanical families.

Although bacterial wilt is particularly seen on potato, tomato, eggplant and capsicum, it is also reported on other crops like bean, ginger, groundnut and anthurium (Dokun *et al*., 2000). *Ralstonia solanacearum* has gained quarantine status and is included in the list of potential bioterrorism agents in USA and other countries in the European Union (Lambert, 2002).

2.1.1 Disease occurrence in India

In India, the disease was first reported on potato by Cappel (1892) in Pune district of Maharashtra and Butler (1903) suggested that the causal organism might be similar to that reported by Smith as *Bacillus solanacearum* in USA. Later in 1941, the disease was reported in tomato by Hedayathullah and Saha, from West Bengal and in chilli from Madhya Pradesh (ICAR, 1969). The disease is endemic in the West coast of India from Thiruvanathapuram in Kerala to Khera in Gujarat and also in central Karnataka, Western Maharasthra and Madhya Pradesh (Shekhawat *et al*., 1978).

Extensive surveys for assessing the severity of bacterial wilt have been conducted by several workers in India. Accordingly in Himachal Pradesh, the disease incidence was the highest in Mandi district and lowest in Solan (Agarwal *et al*., 2006). Seasonal variation in disease severity in solanaceaous crops ranging from one to three per cent in summer to four to 60 per cent in rainy season has been reported by Singh *et al.* (2010). Incidence of bacterial wilt in a wide range of economically important plants like brinjal, tomato, potato, chilli, marigold, ginger, banana, elephant foot yam, jute, tobacco, water melon, bottle gourd, large cardamom, bougainvillea and twelve wild plants namely; *Amaranthus spinosus, Amaranthus viridis*, *Croton sparsiflorus*, *Pennisetum purpurium*, *Malachra capitata*, *Cestrum diurnum*, *Datura metel*, *Solanum indicum*, *Solanum sisymbriifolium*, *Physalis minima*, *Melochia corchorifolia* and *Costus speciosus* in West Bengal was found by Mondal *et al*. (2014). More recently, the disease has been reported from Thane district of Maharashtra (Malshe *et al*., 2016) and also from the lateritic zone of West Bengal (Hembrom *et al.*, 2018).

2.2 Symptomatology

Generally, at the early stages of disease the first visible symptom is the wilting of the youngest leaves. Gradually, the entire plant may wilt quickly and desiccate which is followed by yellowing of foliage and eventually plant death. Vascular bundles of wilt infected tomato appeared as long and narrow with dark brown streaks and this is proceeded by progressive discoloration of the vascular tissue, mainly the xylem, at early stages of infection (Smith 1920). In some cases, the infected plant does not show wilting instead appeared stunted. Further, the leaves of infected plants showed epinasty and adventitious roots appeared on stems (Kelman, 1953). The wilt pathogen primarily invade the xylem vessels and affect the water transport in the host thus leading to wilting (Buddenhagen and Kelman, 1964). Agrios (2005) described that older plant leaves first showed wilting before the youngest leaves or in some cases one side wilting and stunting was seen. Recently wilted plants appeared green and this was a distinct symptom of bacterial wilt along with bacterial ooze as compared to other vascular diseases like Fusarium wilt, which developed yellowing of leaves (Wang and Lin, 2005).

The symptoms of bacterial wilt in tomato include drooping of leaves followed by wilting of whole plants within a few days. Though the infected plants recovered temporarily in the evening, sudden and permanent wilting occurred after a few days (McCarter, 1991). The young plants were affected more and vascular system turned from pale yellow to dark (Gota, 1992). Umesha *et al.* (2005) distinguished infected chilli plants from healthy as infected plants showed a milky white bacterial exudate which was absent in healthy plants.

The potato tubers from the diseased plants showed a brown discolouration (ring) in vascular tissue and eye buds of severely decayed potatoes appeared blackened and at advanced stages, tuber eyes showed grayish brown discolouration (Hingorani *et al.*, 1956). The bacterial ooze from infected tubers (Hingorani *et al.*, 1956) and from stem of the affected potato plants (Mazzucchi, 1995) were the major symptoms observed. Also, lenticel infection was noticed in infected potato tubers in Shimla, Meghalaya and Darjeeling hills (Shekhawat *et al.* 2000). The presence of the pathogen was identified by the bacterial ooze that emerged from the eyes and stem-end attachment of infected potato tubers and when this bacterial exudate dried, a mass of soil adhered to the tubers at the eyes. Also, a creamy fluid exudate usually appeared spontaneously on the vascular ring of the cut surface a few minutes after cutting (EPPO, 2004).

The wilting of infected plants during hot periods of the day and recovery when atmospheric temperature comes down has also been noticed (Champoiseau *et al.*, 2009). In some cases, symptoms are not expressed in plants infected with the pathogen even under typical environmental conditions that are ideal for the pathogen and this is a commonly observed condition called latency (Monther *et al.*, 2010). Bacterial ooze as one of the distinguishing symptom was noticed in eggplants infected with *R. solanacearum* (Oliveira *et al.*, 2014).

2.3 Isolation of the pathogen

As in the case of any other plant pathogenic bacteria, *R. solanacearum* has been isolated on nutrient agar, which appeared as dirty white colonies (Standford and Wolf, 1917). Later, selective media were developed based on the principle of differential growth suppression which improves the efficiency with which a particular species of

bacteria can be isolated. Though nutrient agar was earlier used for the isolation of *R. solanacearum,* numerous selective media were later employed for its isolation. The isolation of the pathogen was performed on Kelman's culture medium with tetrazolium, in which *R. solanacearum* colonies could be identified after cultivation for 48 hours at 28°C (Kelman, 1954). Several other selective media for the isolation of the pathogen has been developed with triphenyl tetrazolium chloride (TZC) forming the basic component. A selective medium consisting of a simplified basal medium incorporating antimicrobial compounds was developed by Karganilla and Buddenhagen (1972). The colony counts of *R. solanacearum* in this media was lower, that is 22-33 per cent as compared to 65-99 per cent reduction in other soil bacteria.

A final selective medium (FSM) was developed for monitoring the population of *R. solanacearum* in naturally and artificially infected soils. Most strains of the pathogen tested on this selective medium were similar in appearance to those observed on TZC agar and could be detected easily (Nesmith and Jenkins, 1979). Chen and Echandi (1982) developed a bacteriocin technique to detect, isolate and quantify *R. solanacearum* in soil. Here, TZC agar was incorporated with chloramphenicol and pentachloronitrobenzene and wide-spectrum bacteriocin-sensitive strains of *R. solanacearum* were used as indicators. The colonies of the pathogen could be identified by clear inhibition zones and the efficiency of recovery of *R. solanacearum* from soil ranged from 92-100 per cent. Hara and Ono (1983) prepared another selective medium by adding antimicrobial compounds to potato sucrose agar and the pathogen was detected in soil containing 10^2 cells g^{-1} dry soil, even in the presence of other crystal violet tolerant bacteria. Another medium (Selective Medium-1) with good plating efficiency and higher selectivity was developed and used to isolate *R. solanacearum* from infested soils, thus eliminating the problem of fungal contamination (Granada and Sequeira, 1983).

Yet another attempt was made by Engelbrecht (1994), who compared modified form of a selective medium developed by Graham and Lloyd (GL), that is, SMSA (Selective Medium South Africa) with the original medium GL and with SM-1 medium for enhancing plating efficiency, reduction of saprophytic soil microorganisms and recovery of *R. solanacearum* from artificially infested soil. The plating efficiency on GL was better than on the other two media, but it did not reduce

other soil microorganisms. Also, recovery rates varied from 36-99 per cent and the colony appearance on SMSA was influenced by other microbes. A modification of this medium, that is, SMSA-E (Selective Medium South Africa - Elphinstone) was proposed by Elphinstone *et al.* in 1996.

An improved selective medium, that is, Potato Crystal violet Chloramphenicol Gellan gum (PCCG) agar was developed for the detection of viable cells of *R. solanacearum* which exhibited higher selectivity (Hara *et al*., 1995). Pontes *et al.* (2017) compared the efficacy of five selective media in literature for the detection of *R. solanacearum* in soil. Among the five, that is, FSM, SM-1, PCCG, SM and SMSA-E media, SMSA-E was found to be the most effective in suppressing the growth of contaminating microorganisms, resulting in the lowest rate of repression and highest recovery rate of the pathogen.

2.4 Maintenance of *Ralstonia solanacearum*

Attempts to preserve viable cultures of *Ralstonia solanacearum* were carried out by several workers. Husain (1958) suspended five loopful of bacterial growth in 5 ml of sterile distilled water in screw capped test tubes and stored them at 25°C. Similarly three to five loopful of bacterial culture was placed into capped test tubes containing 5 ml of sterile distilled water and stored at 22°C for 18-24 months (Kelman and Person, 1961; Khan 1980). Stock cultures of the bacteria were relatively free of avirulent mutant types when stored in sterile distilled water at 22°C (Granada and Sequeria, 1983; Swanepoel and Young, 1988).

The stock cultures of the bacteria prepared in sterile distilled water were stored at room temperatures without affecting the viability of the organism (He *et al*., 1983; Prior and Steva, 1990; Marin and EI. Nashaar, 1993; Mathew *et al*., 2000) whereas Kumar *et al*. (1993) stored the cultures under refrigerated conditions. Black and Sweetmore (1993) reported the storage of culture of *R. solanacearum* in distilled water with backup in 20 per cent glycerol at -80°C. Stead (1993) stored culture of *R. solanacearum* in either freeze-dried condition or on beads at -80°C. Bringel *et al*. (2003) preserved 50 isolates of *R. solanacearum* from potato and aubergine and evaluated the isolates for any variation in biochemical characteristics, pH, carbon and

pathogenicity. They found no alteration in the bacterial characteristics during the preservation period in water.

2.5 Testing the pathogenicity

Pathogenicity is the capability of an organism to cause a disease and the factors responsible for the induction of disease are called pathogenicity factors. Since *R. solanacearum* has got wide host range, pathogenicity tests have been performed in several hosts including solanaceous vegetables employing various techniques. Marin and El-Nashaar (1993) developed a technique of submerging the seedlings of tomato in aqueous inoculum suspension of *R. solanacearum* for 10 seconds and later transplanting them. Whereas the pathogenicity tests of *R. solanacearum* on *Moringa oleifera* was conducted by spraying the bacterial suspension onto pin pricked leaf axil of healthy plants and by dipping the cut end roots of the healthy plants in bacterial suspension (Estelitta *et al.*, 1997).

Vudhivanich (1997) employed a micropipette technique for injection of various concentrations of the inoculum directly into the tomato plant, by inserting diagonally into the stem at the third leaf axil from the top. Whereas, a root severing and root drenching method was used to inoculate capsicum plants, wherein 28 days old seedling roots were injured with a knife by cutting through the soil to one to two centimeters from the collar region and 30 ml bacterial suspension was poured into each pot (Wang and Berke, 1997). Gunathilake *et al*. (2004) performed test pathogenicity on tomato by inflicting damage on root using a sterilized scalpel and 50 ml of prepared culture was introduced to each plant. Wicker *et al*. (2007) tested the pathogenicity of *R. solanacearum* strains on different hosts, namely banana, tomato, sweet pepper, and eggplant. In banana, root scarification was done prior to adding 10 ml inoculum to the soil at four-leaf stage of the plants whereas in solanaceous vegetables inoculation was done at three leaf stage following the similar technique.

Artal *et al.* (2012) evaluated three inoculation methods, *viz.,* soil drenching, leaf clipping and axil puncturing, among this, the soil drenching method of inoculation was found to be the best in all the three crops (tomato, brinjal and chilli) tested, as compared to leaf clipping and axil puncturing methods. The inoculation through soil

drenching recorded significantly highest bacterial wilt incidence in tomato, brinjal and chilli.

Schell (2000) reported that the pathogenicity of *R. solanacearum* is distinctly regulated in early or late stages of infection in response to environmental conditions, such as the presence of host plant and bacterial population densities. Kumar and Sarma (2004) reported that in ginger, wilting started after six days of inoculation when the inoculums was placed in between bottom leaf sheath and pseudo stem. The results of pathogenicity test conducted by Ahmed *et al*. (2013) revealed that all the isolate groups of *R. solanacearum* were able to produce wilt symptom in potato plants incubated by soil inoculation method and were able to produce brown rot symptoms on tubers. On the contrary, the *R. solanacearum* isolate obtained from wilted brinjal plants was not capable of producing any symptom on the potato.

Sharma and Sharma (2014) isolated *R. solanacearum* from brinjal seeds on TZC agar and proved the pathogenicity by seed smothering and stab inoculation of seedlings of tomato, chilli and brinjal. The smothering of brinjal seeds with pure culture of the pathogen resulted in a mortality of 85 per cent and 37.8 per cent in petriplate and water agar test tube method respectively whereas stab inoculation of seedlings resulted in mortality within three days of inoculation. Pathogenicity test in chilli was attempted by soil drenching and detached leaf method and the results indicated that detached leaf method was more efficient as it produced symptoms after one day of inoculation (Shahbaz *et al*., 2015).

2.6 Characterisation of the pathogen

2.6.1 Cultural characterisation

Bacteria exhibit distinguishing characters when cultured on different media and this can be used for their identification by observing the colony morphology which varies with different species. The size, pigmentation, elevation, form, fluidity *etc*. can be used as the criteria for characterisation of different strains.

Standford and Wolf (1917) observed that *R. solanacearum* formed circular, glistening white and slightly raised colonies with smooth margin within 36-48 h of incubation on nutrient agar. Later, Bhide (1948) described the colonies on nutrient agar

as dirty white to brown. Kelman (1954) was the first to describe the colony morphology of *R. solanacearum* mainly into three types by growing the bacterium on triphenyl tetrazolium chloride (TZC) agar. Colonies of normal or wild type were irregularly round, smooth, fluidal and opaque while the mutant type formed round, smooth, butyrous and translucent colonies. Third type formed butyrous translucent colonies with a rough surface.

Khan (1974) described the colonies of four isolates of *R. solanacearum* from chilli, tomato, brinjal and potato on TZC agar. All the isolates except that from brinjal produced fluidal, shiny, slightly convex colonies with slight pink center while that from brinjal produced copious slime and the colony was fluidal. Gnanamanickam *et al.* (1979) observed fluidal colonies of the pathogen isolated from banana on TZC agar which appeared nearly round, smooth with pink center measuring four millimeter in diameter. French *et al.* (1993) used colony phenotype to differentiate mixtures of *R. solanacearum* isolates in Peru and found that strains of biovar III race 1 and biovar II differed consistently in colony size on Kelman's medium with average diameters of 1.8 mm to 2.5 mm and 1.0 mm to 1.5 mm, respectively and they have distinctive formazan pigment patterns. Hussain (1995) reported the presence of purplish pink centered fluidal colonies of *R. solanacearum* on TZC agar from potato in Pakistan. Lemessa and Zeller (2007) found that all the isolates of *R. solanacearum* from Ethiopia produced fluidal and irregular colonies with a red center and whitish periphery on TZC agar whereas on CPG agar it produced larger and whitish fluidal colonies which turned brown after 48 h. of incubation.

Of four different types of colonies of *R. solanacearum* on TZC agar, only the fluidal colonies with raised, light pink center and white periphery were amplified by PCR amplification using the primer. Hence, the primer was suggested for the specific detection of *R. solanacearum* colonies (Singh *et al*., 2010). Narasimha and Srinivas (2012) characterised *R. solanacearum* isolates into virulent and avirulent ones based on colony morphology. The virulent colonies were highly fluidal, white colored with a light pink center and round to irregular margin with 7.0-9.0 mm diameter. On the other hand, the avirulent colonies were round, deep red color with narrow bluish border.

Sahu *et al*. (2013) revealed that the isolates of *Ralstonia solanacearum* from tomato seeds produced raised, circular, smooth colonies which later became irregular with sliming mass while the colonies of *R. solanacearum* on nutrient agar appeared smooth circular, raised and dirty white and the optical feature of the colony was opaque, measuring around three millimeter (Pawasker *et al*., 2014). The cultural characteristics of the *R. solanacearum* was studied three different media *viz.*, glycerol nutrient agar (GNA), King's B agar (KB) and SMSA. The colonies of *R. solanacearum* produced irregularly round or typical, smooth, fluidal, white colonies with or without red centers on glycerol nutrient agar (GNA), tetrazolium chloride (TZC) agar and SMSA (El-Habbaa *et al.,* 2016)

2.6.2 Morphological characterisation

Morphological features of bacteria include shape, size and presence or absence of flagella which can be used in characterisation. Smith (1896) was the first to describe the shape and size of the cells of *Ralstonia solanacearum* as a rod shaped bacterium measuring 0.5 x 1.5 μm. Isolates of the pathogen from tomato, potato, brinjal and chilli were characterised based on size of the cell and they were found to be gram negative, motile with lophotrichous flagella, non-spore forming, and cell size ranged from 1.3 to 1.02 μm x 1.02 to 1.78 μm. (Khan, 1974; Rath and Addy, 1977, Denny and Hayward, 2001).

Khan *et al*. (1980) revealed morphological and physiological characters of *R. solanacearum* chilli isolate and compared with isolates from eggplant, potato and tomato. The pathogen isolated from groundnut and *Moringa oleifera* was gram negative, rod shaped with one polar flagellum and measuring 0.5 to 0.7 μm x1.2 to 2.5 μm in size (Singh and Hussain, 1991; Estellita *et al.,* 1997).

Shoba (2002) performed electron microscopic studies of the pathogen and revealed that it is rod shaped and lophotrichously flagellated with one to three flagella. Narasimha and Srinivas (2012) also studied the cell morphology of the pathogen and found that isolates from tomato were gram-negative, rod-shaped, measuring 0.5-0.7 μm x 1.5-2.0 μm in size, non-capsulated and non-spore forming.

2.6.3 Biochemical characterisation

Biochemical tests are carried out to characterize and group the isolates based on reaction to different tests like citrate utilisation, levan production, glucose oxidation, gelatin liquefaction, nitrate reduction, production of hydrogen sulphide *etc*. Several such tests have been utilised by bacteriologists to characterise *R. solanacearum* (Table 2.1).

Table 2.1 Biochemical characterisation of *R. solanacearum* from different hosts

Hosts	Positive tests	Negative tests	Reference
Potato	Nitrate reduction, citrate utilisation and utilisation of inorganic nitrogen	Gelatin liquefaction, indole production, starch hydrolysis, production of hydrogen sulfide	Bhide, 1948
Groundnut	Hydrogen sulfide production, ammonia production, nitrate reduction, starch hydrolysis and growth in NaCl (2%)	Arginine hydrolase activity	Singh and Hussain, 1991
Potato	Oxidase, catalase and nitrate reduction, solubility in 3 per cent KOH	Gelatin liquefaction, Indole production, starch hydrolysis and production of hydrogen sulfide	Marina and El. Nashaar, 1993
Heliconia and *Musa* sp	Nitrate reduction and glucose utilisation	Tryptophan, arginine dihydrolase activity, hydrolysis of urease gelatinase, and pnitro- β-galactopyranoside	Alvarez *et al.*, 1993
Moringa oleifera	Catalase and nitrate reduction, milk slightly curdled with production of acid	Starch hydrolysis, gelatin liquefaction, sucrose and glucose utilisation, slight inhibition of growth in NaCl (2%) and arginine dihydrolase activity	Estelitta *et al*., 1997
Tomato, Brinjal, Chilli	Catalase and nitrate reduction, starch hydrolysis, glucose oxidation, solubility in KOH, kovacs' oxidase test, lipase activity	Indole production, hydrogen sulfide hydrolysis, fluorescent pigment production, arginine dihydrolase production	James, 2001
Ginger	Solubility in KOH, nitrate reduction, production of catalase and oxidase	Starch hydrolysis, arginine dihydrolase and	Sambasivam and Girija (2006)

	enzymes and fermentation of glucose	hydrogen sulfide production	
Chilli	Kovacs' oxidase test, nitrate reduction test	Gram staining, gelatin hydrolysis, arginine dihydrolase test	Umesha, 2005
Tomato	KOH solubility, kovacs oxidase test, nitrate reduction	Gram staining, levan formation and arginine dihydrolase.	Vanitha *et al.*, 2009
Tomato	Kovacs' oxidase test, catalase test, fermentation of glucose, hydrolysis of tween 80	Gram staining, arginine dihydrolase, levan production, salt tolerance, hydrolysis of gelatin and production of fluorescent pigment.	Chaudhry and Rashid, 2011
Brinjal	Oxidase, catalase, nitrate reduction, nitrate reduction, KOH solubility and citrate utilisation test.	Gram staining, gelatin liquification, starch hydrolysis, indole production, methyl red and voges prouskar tests	Chakravarty and Kalita, 2011
Potato	KOH solubility test, kovacs' test, levan production test and sugar fermentation	Gram staining	Ahmed *et al.*, 2013
Brinjal	Kovacs, test, starch hydrolysis and gelatin hydrolysis	Gram reaction and gelatin hydrolysis	Sharma and Sharma, 2014
Chilli	KOH loop test, catalase test, kovacs' test and oxidation of glucose	Gram staining, levan and lipase production	Shahbaz *et al.*, 2015

2.6.4 Molecular characterisation

Earlier the identification and characterisation of bacteria was based on biochemical characters which is now being replaced by genotypic methods. Among this comparison of bacterial 16S rDNA gene sequence has emerged as a preferred genetic technique as it is the conserved region in the bacterial genome which suggests that random sequence changes can give an accurate measure of evolution (Seal *et al.*, 1993).

Initially based on genetic diversity, Pseudomonads were first categorized into five homology groups on the basis of serology (Morton *et al.*, 1965; Schaad *et al.*, 1978) followed by isozyme pattern (Baptist *et al.*, 1971) and on the basis of rDNA by

Palleroni *et al.* (1973). Further, Currier and Morgan (1981) analyzed genetic diversity of the pathogen using plasmid DNA isolation method. A protocol suitable for the isolation of high molecular weight DNA of *R. solanacearum* was given by Rosenberg *et al.* (1982). Another method to get high quality DNA was suggested by Boucher *et al.* (1987).

Restriction fragment length polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences, known as polymorphisms, in order to distinguish individuals, populations or species. Accordingly, Barlow *et al.* (1988) examined a total of 150 strains of *R. solanacearum* by RFLP analysis and the strains were grouped according to similarity co-efficient and based on this several unknown strains from Heliconia in Australia were identified as race 2. Cook *et al*. (1989) used RFLP analysis to predict the race and biovar of individual strains of *R. solanacearum* and suggested that it can provide a basis for analysis of evolution and geographical distribution of the bacteria. He employed nine DNA probes to study the relationship among 62 strains of the pathogen and based on similarity co-efficient revealed two major divisions: Division I containing all members of race 1 biovars II, IV, and V; and Division II containing all members of biovar I, race 3 and three subdivisions of race 2.

Polymerase chain reaction (PCR) amplification using specific primers is another tool employed to study the bacterial diversity. A PCR amplification of genomic DNA from 112 *R. solanacearum* strains with the tRNA consensus primers T3A and T5A revealed three fingerprint groups which correlated well with the divisions (Cook *et al.,* 1989) made in previous studies (Seal *et al.,* 1992). Another analysis which involved PCR amplification of specific DNA sequences from the 16S rDNA gene using primers OLI 1 and Y2 also confirmed the existence of two divisions of *R. solanacearum* as reported earlier (Cook *et al.,* 1989) and indicated the presence of a third division containing Indonesian strains, which are the closest relatives of blood disease bacterium and *Pseudomonas syzygii* (Seal *et al.,* 1993).

Yabucchi *et al*. (1995) based on analysis of results of phenotypic characterisation, cellular lipid and fatty acid analysis and phylogenetic analysis of 16S rDNA nucleotide sequences transferred *Bulkholderia solanacearum* to a new genus *Ralstonia solanacearum*. Polymerase chain reaction and pulsed-field gel

electrophoresis (PFGR) of genomic DNA of isolates of the *R. solanacearum* race 3 biovar II of Kenya revealed a level of genetic diversity that was previously unrealized (Smith *et al.*,1995). Taghavi *et al*. (1996) determined nearly complete 16S rDNA gene sequences of 19 isolates of *R. solanacearum* and erected dendrogram that revealed two divisions similar to earlier patterns (Cook *et al*., 1989).

Jaunet and Wang (1997) studied the genetic diversity among the biovars of the pathogen which revealed that PCR amplification with different primers displayed great diversity in the population of *R. solanacearum* biovars III and IIIA. Six strain specific DNA probes from three distinct biovars (4, 3 and 2) were developed by Opina *et al.* (1997) which aided in distinguishing these biovars. Further, macro-restriction analysis of genomic DNA and repetitive element sequence-based polymerase chain reaction (rep-PCR) was employed by Smith *et al.* (1998) to detect the genetic diversity amongst 43 European isolates of *R. solanacearum* race 3 biovar 2. Boudazin *et al*. (1999) developed a PCR diagnostic test for detection of *R. solanacearum* at the intraspecific level, based on polymorphisms within the 16S rDNA gene sequence.

Three different molecular methods namely PCR-RFLP analysis of the *hrp* gene region, Amplified fragment length polymorphism (AFLP) analysis and 16S rDNA sequencing were used by Poussier *et al.* (2000) to determine the genetic diversity of *R. solanacearum* strains. The PCR-RFLP analysis, which agreed with the biovar classification, was confirmed by AFLP. It was also revealed that AFLP permitted very fine discrimination between different isolates and was able to differentiate strains that were not distinguishable by PCR-RFLP. Further, Horita and Tsuchiya (2001) made a comparative analysis of the rep-PCR fingerprints of 78 strains of *R. solanacearum*, including six biovars from Japan and various countries and divided these strains into two main clusters. Cluster 1 comprised of biovar III, IV, V, I and some biovar N2 strains from Japan, while cluster 2 included biovar I, II and N2 strains.

Random amplified polymorphic DNA employing molecular markers were used to study the genetic diversity between the strains of *R. solanacearum*. A study on molecular markers to detect the variability in *R. solanacearum* infecting ginger revealed that RAPD analysis using 16 primers showed much diversity among isolates (Sambasivam, 2003). The genetic diversity of *R. solanacearum* assessed using RAPD

method with ten selected decamer primers revealed a higher degree of polymorphism even in the isolates collected from same agro ecological zone (Gunathilake *et al*., 2004). James *et al.* (2006) studied nine isolates of *R. solanacearum* using RAPD analysis performed with 10 decamer primers which showed a high degree of polymorphism among the isolates collected from different agro climatic zones of Kerala. While, 57 isolates of *R. solanacearum* from Karnataka causing wilt on different host plants *viz*., tomato, brinjal, potato, bird of paradise, ginger, chilli, davana and coleus were classified in to seven major clusters based on RAPD analysis (Prasannakumar *et al*., 2013).

Fegan and Prior (2005) proposed a hierarchical classification scheme to reflect the known diversity within the *R. solanacearum* species complex. They classified *R. solanacearum* species complex into four phylotypes based on sequence analysis. Phylotype I included all strains belonging to biovars III, IV, and V which were isolated primarily from Asia and was equivalent to division 1 defined by Cook *et al*. (1989). Phylotype II had equivalency with division 2 (Cook *et al*., 1989), and included strains belonging to biovars I, II and 2T isolated primarily from America and contains race 3 potato pathogen and race 2 banana pathogen. Phylotype III includes strains primarily isolated from Africa and surrounding islands which belong to biovars I and IIT while phylotype IV comprises strains isolated primarily from Indonesia belonging to biovars I, II and IIT. These phylotypes are again grouped into sequevars based on partial endoglucanase gene sequence.

A great deal of diversity among the Indian isolates of *R. solanacearum* was found by phylogenetic analysis of *hrp* gene (Chandrasekhar and Umesha, 2015). Abdurahman *et al*. (2017) based on the results of multiplex PCR and phylogenetic analysis of partial endoglucanase gene sequences, identified all of the isolates infecting potato in Ethiopia as phylotype IIB sequevar 1. Whereas molecular characterisation of 70 strains of *R. solanacearum* from solanaceous crops in Myanmar by rep-PCR DNA fingerprinting revealed seven main clusters at 80% similarity level which suggests that genetic diversity of the strains is endemic (Kyaw *et al*., 2019).

2.7 Detection of races and biovars

The race and biovar classification has gained wide acceptance for subdividing *R. solanacearum*. The different isolates of *R. solanacearum* can be grouped into different races and biovars based on its reaction to differential hosts and utilisation of disaccharides and hexose alcohols respectively. Stanford and Wolf (1917) were the first to study the strain variation of *R. solanacearum* based on cross inoculation method for the isolates of tomato, brinjal, tobacco and potato, but found no difference among the isolates. Further, Kelman and Person (1961) attempted to study the variation among the isolates from tobacco, peanut, tomato, brinjal and Irish potato, but found no success, since the isolates varied from host to host and location to location.

Buddenhagen (1962) classified *R. solanacearum* into races based on host range of individual isolates. According to their studies, race 1 affected tobacco, tomato, many solanaceous, certain weeds, and certain diploid bananas and were usually found in lowland tropics and warm temperate climate while race 2 caused bacterial wilt of triploid bananas (moko disease), heliconia, or both and race 3 affected potato and tomato but is found to be weakly virulent on other solanaceous crops. Further Aragaki and Quinon (1965) proposed a new race of the pathogen infecting ginger designating it as race 4. Also, He *et al.* in 1983 described a new race of *R. solanacearum* from mulberry from China and was designated as biovar 5. Finally, Persley *et al*. (1986) grouped the pathogen based on host range, geographic distribution and the ability to survive in different environmental conditions. According to him, race 1 is the solanaceous strain having a wide host range and distributed throughout tropics and subtropics. Race 2 (Musaceous strain) is restricted to *Musa* spp. and a few perennial hosts initially limited to American tropics but now spreading to Asia. Race 3, the potato strain was restricted to potato and a few alternate hosts in the tropics and sub-tropics. Race 4 (ginger strain) was reported from Philippines and race 5 (Mulberry strain) reported from China on host mulberry.

Hayward (1964) classified *R. solanacearum* into biovars based on oxidation of disaccharides (cellobiose, lactose and maltose) and hexahydric alcohols (dulcitol, sorbitol and mannitol). Biovar I does not oxidise both disaccharides and sugar alcohols, biovar II oxidised only disaccharides, biovar III oxidised both disaccharides

and hexahydric alcohols and biovar IV oxidised only hexahydric alcohols. Biovar V oxidised the disaccharides and sugar alcohols except dulcitol and mannitol.

Zher (1970) studied 35 isolates of *R. solanacearum* following Hayward's scheme. Most of the isolates conformed in character to biovar III and IV, together with a single isolate of biovar I and biovar II. Further, Baptist *et al.* (1971) assigned 12 isolates of *R. solanacearum* into to two biovars. Biovar I included representative of Hayward's biovar I and II, except two strains of the former, which together with the representatives of biovar III and IV were included in biovar II. After comparison of the virulence of isolates from tomato, aubergine and potato collected from different parts of India on differential hosts and by carbohydrate utilisation tests Rao (1976) concluded that race 1 biovar III to be prevalent. Rath and Addy (1977) noted that 10 isolates of *R. solanacearum* causing wilt in tomato to be race 1 based on pathogenicity tests. The prevalence of biovar III in Philippines was reported by Tabei and Quimio (1978). The different strains of *R. solanacearum* from 10 countries in North, Central and South America from four hosts (potato, tobacco, tomato and capsicum) were tested for carbohydrate utilisation and revealed the presence of biovar I, II and III (Martin *et al*., 1982).

Further, the isolates of race 3 correspond to biovar II but the reverse is not always true (Buddenhagen, 1985). The studies of Sinha (1986) revealed race 1 and 3 and biovar II, III and IV to be prevalent in India. Within biovar II, two phenotypes were present: phenotype A (biovar 2T) comprising strains metabolically more active, adapted to warm tropical conditions and with a wider host range whereas phenotype B (biovar 2A) included metabolically less active strains adapted to cool temperature and corresponds to race 3 (Hayward, 1991). Kumar *et al.* (1993) studied 12 isolates of *R. solanacearum* from tomato, capsicum, bell pepper, and brinjal collected from Himachal Pradesh. Six of the seven tomato isolates were identified as biovar III, whereas one isolate differed for not utilising dulcitol and was designated as biovar III A, a sub-type of biovar III.

The isolates of *R. solanacearum* collected from eggplant, tomato and potato from West Bengal belonged to race 1 biovar III of *R. solanacearum* (Chakrabarti *et al.*, 1995). The variability tested among 28 strains of *R. solanacearum*

from different commercial tomato fields in Brazil indicated that 7 strains were biovar I and 21 strains were classified as biovar III (Silveira *et al.,* 1998). The isolates from potato, tomato, groundnut and Bird-of-paradise were classified as race 1 and biovar III based on pathogenicity tests and Hayward's classification, respectively (Venkatesh, 1999). The strains of *R. solanacearum* isolated from bacterial wilt potato tubers grown in Nepal were race 3, biovar II as observed by Dhital *et al*. (2001).

Further, Kumar *et al*. (2004) confirmed the predominance of biovar III in India by testing the carbohydrate utilisation of thirty-three strains of *R. solanacearum* from ginger, paprika, chilli, tomato, and potato from Kerala, Karnataka, West Bengal and Assam. James *et al*. (2006) classified *R. solanacearum* collected from different solanaceous crops from different agro climatic zones of Kerala as biovar III and IIIA and race 1 and 3. Whereas 54 isolates of the pathogen collected from different agro climatic zones of Karnataka were classified as race 1, biovar III, and 3 isolates as race 1, biovar III B (Chandrashekara *et al.,* 2012). Ahmed *et al*. (2013) designated the *R. solanacearum* isolates as biovar III and race 3 responsible for bacterial wilt of potato in Bangladesh. Further, the strains of *R. solanacearum* causing bacterial wilt of solanaceous crops in Karnataka were grouped into race 1 and 3 and biovar II and III (Kumar *et al*., 2017).

2.8 Ecology of *Ralstonia solanacearum*

Being a soil borne pathogen, the survival of *R. solanacearum* depends on the initial inoculum in soil and the ability to tide over the adverse conditions. However, the absence of a suitable host, forces the pathogen to lead a saprophytic existence in the soil and plant debris. (Schuster and Coyne, 1974).

2.8.1 Survival of *Ralstonia solanacearum* in soil

The survival of *R. solanacearum* in soil depends on soil temperature, moisture, pH, aeration, competition and antagonism. Survival of the pathogen up to two and half years in the soil has been reported by Coleman (1909) but the survivability varied with soil (Kelman, 1953). Accordingly the persistence of the bacterium varied from 16 months in pot soil (Das and Chattopadhyay, 1955) to 250 days in free-state in soil or

in the infected plant parts (Rangaswami and Thirunakarasu, 1964). However, it could survive up to six years as a free propagule in bare soil (Crosse, 1968).

The survival of *R. solanacearum* (race 3) was enhanced in deeper soil layers due to less temperature fluctuation and a lower degree of predation by protozoa or antagonism by the indigenous microbiota (Graham and Lloyd, 1979). Even though the pathogen persisted in soil for two years, a decline in the population of pathogen with time has been noticed by Shekhawat *et al*. (1978). Further, Granada and Sequeira (1983) highlighted that the survival of *R. solanacearum* in soil varied with race of the pathogen and found that race 1 survived in the soil longer than race 2 and race 3. Though survival of *R. solanacearum* biovar 2 declined progressively over time, the pathogen persisted for periods of 10 to 12 months in soil (Elsas *et al*., 2000).

2.8.2 Influence of soil physico-chemical properties on the survival of *Ralstonia solanacearum*

Ralstonia solanacearum, being a soil-borne pathogen, its survival in soil is influenced by physico-chemical properties of soil such as soil moisture, soil temperature, soil type, pH and nutrient availability. The occurrence and epidemics of bacterial wilt are tightly correlated with the physical and chemical properties of soil (Li *et al.*, 2017).

2.8.2.1 Influence of soil physical properties on *Ralstonia solanacearum*

The presence of the bacterium varies with soil depth (Graham and Lloyd, 1979), with soil type (Shekhawat *et al.*, 1978) and with soil moisture and temperature (Shekhawat and Perombelon, 1991). The soil temperature is an important environmental factor that affects *R. solanacearum* in multiple plant patho-systems and their interactions with their hosts (Hayward, 1991)

Soil temperature plays an important role in the survival and multiplication of the pathogen in soil. The pathogen favoured a temperature ranging from 25-37 °C for growth and multiplication with the minimum being 10 °C and maximum being 41 °C. Temperature also played an important role in the geographic distribution of *R. solanacearum*, since it was rarely found in areas where the mean temperature falls below 10 °C in midwinter months (Kelman, 1953). The disease occurrence in

susceptible cultivars of crops was found to be enhanced at a temperature range of 30-35°C (Quinon *et al*. 1964). Accordingly, the disease severity caused by *R. solanacearum* was found to be significantly greater at 32.2°C than at 26.6°C in resistant tomato cultivars (Krausz and Thurston, 1975). Further, the temperature requirement for disease development and reproduction of the pathogen is distinct for different races of the pathogen (Swanepoel, 1990) and hence *R. solanacearum* race 3 biovar 2 isolates required a lower optimum growth temperature (approximately 27°C) (Stansbury *et al*., 2001). Soil temperature also has a profound influence on the virulence of the pathogen, that is, the pathogen maintained its virulence best in soil at 30° C (Shekhawat and Perombelon, 1991) and decreased virulence was observed at temperatures above 35°C and below 10°C (Stansbury *et al*., 2001). So cool temperate climate is characterized by low incidence of the disease as soil temperatures below 20°C are not suitable for the disease development (Gadewar *et al*., 1999). Thus, the disease was generally limited to areas without frozen soils, and was particularly severe in tropical and subtropical areas (Agrios, 2005).

Apart from soil temperature, soil types have a profound influence on bacterial wilt incidence. The finer-textured soils were more conducive for the growth and multiplication of *R. solanacearum* (Nesmith and Jenkins, 1979; Elsas *et al*., 2000). The soil types in combination with different moisture levels, significantly influenced the severity of bacterial wilt in groundnut (Abdullah, 1983). So an elevated disease level was expressed in clay soils with high water-holding capacities as sandy loam soil with a high sand content and low silt or clay content, with low water-holding capacity, was unfavorable for the pathogen and wilt incidence (Keshwal *et al*., 2000).

Soil moisture is another important parameter which influences bacterial wilt incidence. Dry soil conditions reduced survival of the pathogen in soil and resulted in a decreased viability of the bacterium (Buddenhagen and Kelman, 1964). Thus, the bacterial wilt of brinjal was more severe during heavy monsoon, when fields become frequently waterlogged as observed by Das and Chattopadhyay (1955). Similar to soil temperature, the disease occurrence in susceptible cultivars was often favored by high moisture (Quinon *et al*. 1964). High soil moisture conditions enhanced the spread of the pathogen, particularly when root systems are intermingled (Kelman and Sequeira, 1965). Moreover, the population of the pathogen declined at moisture levels below

field wilting point (Ramos, 1968). Thus, the most favorable soil moisture for the survival and reproduction of the pathogen was found to be -0.5 to -1 bar whereas -55.0 to -15.0 bar was unfavorable (Nesmith and Jenkins, 1983).

Hayward (1991) reported that the bacterium is destructive in moist soils as the virulence of the pathogen is aggravated in soils having 20-40% of water holding capacity (Shekhawat and Perombelon, 1991). Alike soil temperature, soil moisture influences the survival of different races of the pathogen as the survival of *R. solanacearum* race 2 was enhanced by high water content in soil (Elsas *et al*., 2000). Also, severe drought had a negative impact on population densities of the bacteria and soils with low water-holding capacity, was unfavorable for the pathogen and wilt incidence (Keshwal *et al*., 2000). Though bulk density of the soil is not found to have a direct influence on the population of *R. solanacearum*, the availability of various nutrients especially Ca in soil is negatively correlated to bulk density which predisposes the plants to bacterial wilt (Chaudari *et al*., 2013). Also, the soils with low bulk density supports the growth and multiplication of beneficial microflora which in turn suppresses the pathogen and hence the disease (Li *et al.,* 2017).

Further, Gupta *et al*. (2018) explained that low moisture content in the soil reduces rhizospheric inoculum of *R. solanacearum* in soil and drought stress-inflicted structural alterations, such as reduced cortical cell size hinders pathogen entry in the host plant. Further, host plant exhibited inhibited transpiration pull under low soil moisture regimes, which restricted the longitudinal movement of the pathogen in plant which restricts its spread within the plant.

2.8.2.2 Influence of soil chemical properties on *Ralstonia solanacearum*

The incidence of the soil borne diseases is exacerbated under conditions of low soil fertility and low pH (Woltz and Jones, 1968; Shekhawat *et al.*, 1978; Bailey and Lazarovits, 2003). A lower soil pH favoured bacterial wilt (Shekhawat *et al*., 1978). However, under laboratory conditions, soil amendments had little effect on the population of the pathogen but the amendments influenced the total microbial population in general (Devi *et al*., 1981). Also, soils with lower pH and organic matter contents were conducive to bacterial wilt as it favoured the multiplication of the pathogen (Ramesh and Bandyopadhyay, 1993). Also, an increase in disease resistance

was seen in susceptible plants treated with Ca or certain formulations of N (Elphinstone and Aley, 1993). Prior *et al*. (1993) attempted to the control of bacterial wilt in two contrasting soil types by management of water in irrigated vertisols and supply of organic amendments in oxisol soils. In the latter, large nitrogen inputs greatly reduced the severity of bacterial wilt throughout several successive tomato crops whereas in former minimal guidelines for water management was defined to maintain their effective natural suppressive properties.

A soil amendment composed of urea and CaO caused a significant reduction of pathogen population in soil which coincided with an increase in pH and nitrate accumulation in soil (Michel and Mew, 1998). The suppressive effects of pH and nitrate on the growth of the pathogen was further confirmed by *in vitro* experiments which concluded that pre-planting soil amendment consisting of urea and CaO significantly reduced the pathogen population and tomato bacterial wilt. They proposed that the effect of soil amendments seemed to be linked with pH as the population of the pathogen was effectively reduced when the pH was close to 7. Further, bacterial wilt of tomato was found to be suppressed in poultry manure and FYM added soils which contained a higher amount of water soluble organic carbon and nitrogen and a lower C/N ratio (Islam and Toyota, 2004). Apart from the pathogen population, the diversity and composition of bacterial and fungal communities in soil also have been influenced by pH, N content, P content and ratio of cations(Ca^{2+}, Mg^{2+}, and Al^{3+}) in the soil (Chaparro *et al.*, 2012).

The observations on healthy and diseased soils induced by prolonged potato monoculture came to the conclusion that soil organic matter was the key variable associated with healthy soils whereas NH_4^+-N and EC were the key variables associated with diseased soils (Lu *et al*., 2013). Further, an integrated treatment comprising of lime, ammonium bicarbonate and bio-organic fertilizer could significantly suppress bacterial wilt in field as the treated soil had a higher pH and a higher amount of available K and the abundance of *R. solanacearum* were significantly lowered in these soil (Wu *et al*., 2014). Similar results were obtained by Zheng *et al*. (2014) and Liu *et al.* (2015) where the soil pH value, electric conductivity, organic carbon, NH_4^+-N, NO_3^--N and available K content were positively related with soil suppressiveness and negatively correlated with the population of *R. solanacearum*.

Also, pH, organic carbon and calcium showed significantly negative correlation with wilt infection rate (Yang *et al*., 2017). A comparison of bacterial wilt infected soils to healthy soils revealed higher soil pH, available phosphorous and potassium content the healthy soils (Wang *et al.*, 2017).

2.8.3 Influence of rhizosphere microflora on *Ralstonia solanacearum*

The density and diversity of rhizosphere microflora comprising of fungi, bacteria, actinomycetes and pseudomonads have a profound influence on disease suppression and soil health. The survival of *R. solanacearum* in soil is often influenced by the rhizosphere microflora through competition and antagonism and many attempts have been taken up to understand the influence of rhizosphere microflora on the plant pathogen.

The effect of rhizosphere microflora of sorghum on the survival and growth of *R. solanacearum* in artificially inoculated soils was studied by Balasubramanian (1972). The population of pathogen was much lower in the non-sterile soils inoculated with the pathogen which can be attributed to the increased competition and antagonism exerted by rhizosphere microflora when compared to sterile soil. On comparing the pathogen population in the rhizosphere of healthy and wilted plants of brinjal, the pathogen population was found to be higher in the latter. The virulent population in rhizosphere of the wilted plants was 4 to 50 folds higher than the avirulent population whereas the avirulent population was 1 to 20 folds higher in the rhizosphere of non-wilted plants than that of wilted plants (Gowda *et al.*, 1974).

The application of bioorganic fertilizer altered microbial community structure (Mazzola, 2007) and a significant increase in the bacterial and actinomycetes populations and a significant reduction the population of *Ralstonia solanacearum* were observed by Liu *et al*. (2015). Further investigations carried out by Yang *et al.* (2017) revealed the rhizosphere soil microbial community diversity is positively correlated with plant health and bacterial operational taxonomic units (OTUs) were more diverse in fields with healthy plants than in fields with infection. In fields with healthy plants, the major taxa were mainly *Bacillus* and several members in the phylum *Actinobacteria* which are often considered as plant-beneficial microbes.

Wang *et al*. (2017) demonstrated that healthy soils exhibited higher microbial diversity than the bacterial wilt infected soils. More abundant beneficial microbes including *Bacillus, Agromyces, Micromonospora, Pseudonocardia, Acremonium, Lysobacter, Mesorhizobium, Microvirga, Bradyrhizobium, Acremonium* and *Chaetomium* were found in the healthy soils rather than the bacterial wilt infected soils. This is supported by the observations of Qi *et al*. (2019) that the composition and structure of the microbial network of the bacterial wilt susceptible soil were different from that of the healthy soil and many microbial network connections were missing in the bacterial wilt susceptible soil, which most likely provided conditions leading to higher rates of bacterial wilt disease.

Thus *R. solanacearum* being a successful soil inhabitant is influenced by the physical, chemical and biological properties of soil which may further contribute its highly variable nature. Soil microbiome engineering, a relatively new field can be successfully employed to alter the soil microbiota, thus resulting in the establishment of a more diverse and balanced soil microbial community which leads to improvement in soil health and disease suppressiveness.

3. Materials & Methods

3. MATERIALS AND METHODS

The experiments on 'Characterisation of *Ralstonia solanacearum* (Smith) *Yabuuchi et al*. infecting solanaceous vegetables in relation to physico-chemical and biological properties of soil' was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara during 2018-2019. The study was based on four different agro ecological units of Kerala and involved isolation as well as characterisation of the isolates of the pathogen obtained from different locations. Rhizosphere soil samples of healthy and diseased plants were also collected and the effect of various soil properties on the disease were studied.

3.1 SURVEY AND COLLECTION OF BACTERIAL WILT SAMPLES

Purposive sampling survey was conducted during the period from March to November, 2018 to collect bacterial wilt affected plant samples of solanaceous crops. The samples were collected from four different agro ecological units (AEUs) of Kerala, *viz.*, North Central laterite (NCL), Marayur hills (MH), Southern laterite (SL) and Palakkad central plains (PCP) (Table 3.1). The wilted plant samples were collected from two locations in each AEU. The per cent disease incidence (PDI) of bacterial wilt in these locations was calculated using the formula

$$\text{PDI} = \frac{\text{Number of plants wilted X 100}}{\text{Total number of plants}}$$

Table 3.1. Details of survey locations along with GPS coordinates

Sl No.	Location	Code assigned	Crops	GPS coordinates
1.	Pudukkad	NCL 1	Brinjal	10° 25´ 15.87´´N 76° 14´ 35.102´´E
2.	Vellanikkara	NCL2	Tomato	10° 33´ 6.037´´N 76° 17´ 5.413´´E
3.	Perumalai	MH 1	Potato	10° 12´ 19.541´´N 77° 11´ 26.188´´E
4.	Kanthaloor	MH 2	Potato	10° 12´ 14.998´´N 77° 11´ 26.073´´E
5.	Pravachambalam	SL 1	Brinjal	8° 23´ 47.251´´N 77° 0´ 14.356´´E
6.	Venganoor	SL 2	Brinjal	8° 24´ 16.279´´N 77° 0´ 57.344´´E

7.	Nemmara	PCP 1	Brinjal	10° 36´ 22.172´´N 76° 35´ 36.992´´E
8.	Vithanassery	PCP 2	Brinjal	10° 36´ 28.098´´N 76° 38´ 3.155´´E

Rhizosphere soil samples of healthy and diseased plants were also collected separately. Randomly selected wilted plants were subjected to ooze test in the field to confirm the presence of bacteria. Thus, diseased plant samples were collected from eight locations and 16 soil samples were taken from the rhizosphere of diseased and healthy plants of these locations.

3.2 ISOLATION AND PATHOGENICITY TEST

The bacterium associated with the wilted plants collected during the survey was isolated and pathogenicity test was proved by inoculation on healthy plants.

3.2.1 Isolation of the pathogen

Basal stem portion of the wilted plants were washed in water, then surface sterilized with 70 per cent alcohol and cut into small pieces of 10-15 mm in length using a sterile blade. These were then kept suspended in 5 ml of sterile water for 15-20 minutes. When the water became turbid with bacterial ooze, a loop full of the suspension was streaked on triphenyl tetrazolium chloride (TZC) agar (Kelman, 1954), which is the selective medium for the isolation of *Ralstonia solanacearum*. The plates were incubated at room temperature for 48 h. Composition of TZC agar is given in Appendix I.

3.2.2 Pathogenicity test

Fresh bacterial suspension collected from wilted brinjal, tomato and potato plants were adjusted to the concentration of OD_{600} = 0.3 nm and inoculated into three weeks old seedlings of susceptible varieties of brinjal (Pusa Purple long), tomato (Pusa Ruby) and potato (Kufri Jyothi) at 15-20 cm height. Further, a loop full of the suspension was plated on TZC agar to confirm the association of bacterium. The brinjal and tomato plants were inoculated by root dip method (Chandrashekara *et al*., 2012) whereas potato plants were inoculated by stem stab method (Bakade and Sagar, 2012). Inoculated plants were incubated under plastic covers and observed daily for

wilting symptoms up to 15 days. Wilted plants were subjected to ooze test for the confirmation of the infection and the bacterial suspension collected from the plants was plated on TZC agar to confirm the presence of bacteria.

3.2.3 Purification and maintenance of the isolates

Round to oval fluidal single colonies with red or pink center typical of *R. solanacearum* selected from TZC agar were suspended in sterile distilled water (1 ml) and then purified by quadrant streaking on casaminoacid peptone glucose (CPG) agar (Appendix I). Three to five characteristic single colonies were picked and suspended in sterile distilled water (5 ml) taken in screw-capped glass vials and stored at room temperature for further use. The isolates were streaked on TZC agar once in two months to check the virulence of the isolates.

3.3 QUANTIFICATION OF POPULATION OF THE PATHOGEN IN RHIZOSPHERE

The enumeration of pathogen population in rhizosphere soil of diseased and healthy plants collected from eight locations was carried out by serial dilution and plating technique on Selective Medium South Africa – Elphinstone (SMSA-E) (Elphinstone *et al*., 1996) (Appendix I) The population of pathogen was determined as number of colony forming units per gram (cfu g^{-1}) of soil.

3.4 CHARACTERISATION OF THE ISOLATES

The isolates of bacterial wilt pathogen were characterised based on cultural, morphological and molecular characters.

3.4.1 Cultural characterisation

The cultural characters of the isolates were observed by streaking them on TZC agar. Suspension of each isolate was prepared by suspending a loop full of inoculum in sterile water (1 ml) taken in centrifuge vials. A loopful of the suspension was then streaked on TZC agar and incubated to obtain single discrete colonies. The colony characters *viz.,* size, pigmentation, form, margin, mucoid and elevation were studied as per Bergey's manual for the cultural characterisation of the isolates (Table 3.2).

Table 3.2 Colony characters for bacterial characterisation

Sl. No.	Colony characters	Description
1.	Size	Pinpoint
		Small
		Moderate
		Large
2.	Pigmentation	Colour of the colony
3.	Form	Circular- Unbroken peripheral edge
		Irregular- Indented peripheral edge
		Rhizoid- Root-like spreading growth
4.	Margin	Entire- Sharply defined, even
		Lobate- Marked indentations
		Undulate- Wavy indentations
		Serrate- Tooth-like appearance
		Filamentous- Thread-like, spreading edge
5.	Elevation	Flat- Elevation not discernible
		Raised- Slightly elevated
		Convex- Dome-shaped elevation
		Umbonate- Raised with elevated convex central region
6.	Fluidity	Fluid
		Non- fluid

3.4.2 Morphological characterisation

3.4.2.1 Gram staining

The isolates of *R. solanacearum* were morphologically characterised based on Gram's reaction.

3.4.2.2 Scanning electron microscopy

The bacterial cell morphology was observed using scanning electron microscope (Tescan Vega-3 LMU) at Central Instrumentation Laboratory, Kerala Veterinary and Animal Sciences University, Mannuthy. The protocol used was slightly modified from the protocol described by Li *et al.* (2016).

Protocol

A loopful of bacterial cells suspended in 1.5 ml of sterile water

↓

Centrifuged at 6000 rpm for 5 min.

↓

Supernatant removed and added 1.5 ml of PBS (pH=7)

↓

Centrifuged at 6000 rpm for 5 min.

↓

Supernatant removed and added 1.5 ml of glutaraldehyde (2.5%)

↓

Incubated at 37°C for 1.5 h. with intermittent vortexing

↓

Washed three times with PBS (pH=7)

↓

Serial dehydration with ethanol (30%, 70%, 80% & 90%) and incubated for 10 min.

↓

Added 1.5 ml of 100% ethanol, vortexed and incubated for 1h.

↓

Centrifuged at 6000 rpm for 5 min.

↓

Supernatant discarded and air dried the sample

↓

Sample smeared on stub and was sputter coated with gold

↓

Stub loaded into the scanning electron microscope

The optimum working distance of the electron beam for obtaining high resolution images of the bacteria was standardized by trial and error method. Working distance of 17.85 mm and 8.98 mm giving magnifications of 16.2 kx and 44.9 kx respectively, yielded high resolution images of the bacterial cells and SEM micrographs were taken.

3.4.3 Molecular characterisation

Molecular characterisation of the bacterial isolates were carried out by PCR amplification followed by sequencing of 16S rDNA gene. The genomic DNA isolation of the bacterial isolates and PCR amplification of 16S rRNA gene was carried out at Molecular laboratory, Department of Plant Pathology. The amplicons were purified and sequenced using the automated sequencing facilities at Agri Genome Lab Pvt. Ltd., Kochi and Rajiv Gandhi Center for Biotechnology, Thiruvananthapuram.

3.4.3.1 Isolation of genomic DNA

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

Protocol

1. A 48 h. old single colony of the bacteria was grown on CPG agar and was inoculated into 100 ml of sterilized CPG broth.
2. The inoculated broth was incubated overnight (12 h.) in an orbital shaker (150 rpm) at room temperature.
3. The broth (2 ml) was taken in micro centrifuge tube and centrifuged at 10,000 rpm for 10 min. at 4°C.
4. The supernatant was decanted and the pellet was washed twice with 1.5 ml of NaCl (1%) to remove extra cellular polysaccharides.
5. The cells were then suspended in 875 µl of TE buffer into which 100 µl of SDS and 5 µl of proteinase K were added and incubated at 37°C for 1h.
6. After incubation, equal volume of phenol-chloroform mixture was added and incubated for 5 min.
7. The contents were then centrifuged at 10000 rpm for 10 min at 4°C. After centrifugation, three layers were observed. Top aqueous layer containing DNA, a middle layer with cell debris and a bottom layer containing phenol.

8. The top aqueous layer was carefully pipetted out and transferred in to a fresh tube and the process was repeated once again.

9. After centrifugation, the supernatant was collected and 100 µl of 5 *M* sodium acetate was added and mixed gently.

10. Isopropanol (2 ml) was added and mixed gently by inversion till DNA precipitates as white thread-like strands.

11. The reaction mixture was then incubated at -20 °C for 2 h.

12. After incubation, it was centrifuged at 10000 rpm at 4°C for 10 min. and the supernatant was discarded carefully.

13. The DNA pellet was then washed with 70 per cent ethanol, followed by washing with 100 per cent ethanol.

14. The DNA pellet was then air dried and dissolved in distilled water.

3.4.3.2 Qualitative analysis of DNA using agarose gel electrophoresis

The quality of the DNA obtained was assessed by agarose gel electrophoresis.

Protocol

1. Agarose solution (0.8%, 100 ml) was prepared by dissolving 0.8 g of agarose in 100 ml 1X TAE buffer. The solution was allowed to cool to 42 °C and two drops of ethidium bromide was added and mixed well.

2. Gel casting tray was wiped with 70 % ethanol and warm agarose solution was then poured. The solution was allowed to solidify for 40-45 min.

3. After solidification, the casting tray was placed in the electrophoresis unit filled with 1X TAE buffer in such a way that the gel is immersed in the buffer up to a depth of 1 mm. The casting tray was positioned in such a way that the wells were towards cathode.

4. Sample (5 µl) was mixed with loading dye (1 µl) and this mixture was loaded into the wells except the first well to which one Kbp ladder was added.

5. Electrophoresis was carried out at 80 V till the dye has migrated to two third of the gel and the bands of DNA were visualized using gel documentation system (GeNei TM- UVITEC Fire Reader, Merck, UK + Dell computer system).

3.4.3.3 Quantification and purity analysis of genomic DNA

The quality and quantity of DNA was detected by recording the concentration and absorbance of samples using Nano drop 1000 spectrophotometer (Thermo Scientific, USA). The arm and measurement pedestal of the equipment was cleaned using sterile tissue paper and was calibrated with 1 µl distilled water as blank. The concentration and quality of the DNA samples were recorded by loading 1 µl of the sample to the loading arm. The arm and measurement pedestal of the equipment was wiped using sterile tissue paper before reading each sample to prevent sample carryover in successive measurements. The absorbance was recorded at 260 nm and 280 nm wavelength and purity was indicated by the ratio $A_{260/280}$. An absorbance value which lies between 1.8 to 2.0 indicates the purity of DNA whereas a value lower than 1.8 indicates protein contamination and a value higher than 2.0 indicates RNA contamination.

3.4.3.4 PCR amplification of 16S rDNA

The PCR reaction was carried out to amplify the 16S rRNA gene using the universal 16S rRNA primer sets 8 F and 1522 R (Frank *et al.*, 2008).

Table 3.3 Primers used for 16S rDNA gene amplification

Primer	Sequence 5'-3'	Length in bp
8 F	AGAGTTTGATCCTGGCTCAG	20
1522 R	AAGGAGGTGATCCAGCCGCA	20

(a) Composition of PCR mixture

Composition of mixture used for PCR reaction mixture is given in Table 3.4. The reaction mixture was gently mixed using a mini spinner and then the reaction was carried out.

Table 3.4 Composition of PCR mixture

Component	Per reaction volume required
Master mix	12.5 µl
Template DNA	2.0 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
dH_2O	9.5 µl
Total	25.0µl

(b) Standardization of PCR conditions

PCR was carried out in PCR thermo Cycler (Eppendorf Master Cycler) using universal primers. The concentration of template DNA to be taken and annealing temperature were standardized. The reaction was carried out at four different template DNA concentrations *viz*., 10 ng/µl, 30 ng/µl, 50 ng/µl and 100 ng/µl and twelve different annealing temperatures in the range of 47°C to 61°C for standardization. The best concentration of DNA template and annealing temperature were selected based on the quality of band obtained in agarose gel electrophoresis.

PCR amplification profile

The selected PCR conditions which were used in the present study are given below.

Concentration of DNA template - 30 ng/µl

Initial denaturation - 95°C for 3.0 min.

Denaturation - 94°C for 1.30 min.

Annealing - 55°C for 0.40 min. } 34 cycles

Primer extension - 72°C for 1.30 min.

Final extension - 72°C for 20.0 min.

Final hold - 4°C for 10.0 min.

3.4.3.4.1 Analysis of PCR products by gel electrophoresis

The PCR products were analyzed in agarose gel (1%) prepared using 1X TAE buffer with 0.5 µg/ml of ethidium bromide. The PCR products (5 µl) were loaded into the wells along with one Kbp ladder to compare the size of the product. Electrophoresis was carried out at 80V till the dye moved three fourth of the casting tray. The amplified product was visualized and the image was documented using gel documentation system (GeNei TM- UVITEC Fire Reader, Merck, UK + Dell computer system).

3.4.3.4.2 Sequencing of 16S rDNA

The amplicons obtained were purified and sequenced using the automated sequencing facility at Agri Genome Lab, Kochi and Rajiv Gandhi Center for Biotechnology, Thiruvanathapuram. The sequence information of the isolates were further analyzed using various bioinformatic tools for the characterisation of the bacterial isolates.

3.4.3.5 In-silico analysis of sequences

In silico analysis using nucleotide basic local alignment search tool (BLASTn) software, was used to find out the homology of the nucleotide sequences. The sequences obtained for the isolates of *R. solanacearum* acted as the query sequence. The query sequence was compared with already existing sequences in the NCBI database and the sequences which were well aligned with the query sequence were identified.

3.4.3.6. Phylogenetic analysis

Phylogenetic analysis of the 16S rDNA sequences of the eight isolates were performed. The sequences of the isolates were aligned with 21 other *Ralstonia solanacearum* isolates retrieved from the NCBI database using Clustal W program available in the MEGA X software. The phylogenetic analysis of thus aligned sequences was conducted using MEGA X software and the phylogenetic tree was constructed using Neighbor-joining method (Saitou and Nei, 1987).

35 IDENTIFICATION OF RACES BY PATHOGENICITY ON DIFFERENTIAL HOSTS

The race identification was done was by artificial inoculation on differential hosts (Table 3.5) as suggested by Buddenhagen *et al.* (1962), Aragaki and Quinon (1965) and He *et al*. (1983).

Table 3.5 List of races and corresponding host differentials for *R. solanacearum*

Races	Differential hosts	Reference
Race 1	Solanaceous vegetables	Buddenhagen *et al*.(1962)
Race 2	Triploid banana	
Race 3	Potato and tomato	
Race 4	Ginger	Aragaki and Quinon (1965)
Race 5	Mulberry	He *et al*. (1983)

The reaction of specific host differentials to *R. solanacearum* isolates was tested. The details of the crops used as differential hosts are given in Table 3.6. These plants were raised sterilized potting mixture containing soil, coir pith and FYM in the ratio of 1:1:1.

Table 3.6 List of host differentials used in the study

Sl No.	Host differentials	Scientific name	Variety
1.	Tomato	*Solanum lycopersicum* L.	Pusa Ruby
2.	Brinjal	*Solanum melongena* L.	Pusa purple long
3.	Chilli	*Capsicum annum* L.	Pusa Jwala,
4.	Banana	*Musa paradisiaca*	Nendran
5.	Potato	*Solanum tuberosum*	Kufri jyothi
6.	Ginger	*Zingiber officinale*	Athira
7.	Mulberry	*Morris alba*	V1

3.5.1 Inoculation on solanaceous crops for detection of race 1

Three weeks old tomato, chilli and brinjal seedlings were uprooted gently, washed free of soil and a few tertiary roots were clipped with sterilized scissors and dipped in bacterial suspension for 30 minutes. The inoculated seedlings were transplanted into sterilized potting mixture (Chandrashekara *et al*., 2012).

3.5.2 Inoculation on banana and mulberry for detection of race 2 and 5

In-situ inoculation was carried out for banana (at four leaf stage) and mulberry (three to four week old cuttings) by scrapping off the surface soil to expose roots and 20 ml of bacterial suspension was poured around the roots and covered back with soil (Shoba *et al.,* 2002).

3.5.3 Inoculation on potato for detection of race 3

In the case of potato, the inoculation was done by following the protocol given by Bakade and Sagar (2012). The plants were inoculated by stem stab method when they were 15-20 cm tall. The 3rd and 4th axil bud from the top is pierced using a sterile needle and inoculated by placing a droplet of suspension (15-20 µl) on injury.

3.5.4 Inoculation on ginger for detection of race 4

Ginger plants were inoculated by leaf axil puncturing method when they were 45 days old. The 3rd and 4th leaf axils of the plants were punctured and a piece of cotton soaked with bacterial inoculum was placed at these leaf axils (Vijayaraghavan, 2007).

All the plants inoculated were then covered with polythene bags and incubated. The plants inoculated with sterile water served as control. The plants were observed for symptoms for 7-15 days after inoculation. The isolates were categorized into different races based on the reaction of differential hosts on artificial inoculation.

3.6 IDENTIFICATION OF BIOVARS BY DISACCHARIDE AND SUGAR ALCOHOL UTILISATION TEST

The eight isolates were differentiated into biovars based on utilisation of disaccharides and sugar alcohols (Hayward, 1964).

Table 3.7 Key for identification of biovars of *R. solanacearum*

Utilisation of	Biovar				
	I	II	III	IV	V
Cellobiose	-	+	+	-	+
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
Dulcitol	-	-	+	+	-
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-

The basal medium was prepared as described by Hayward (1964) (Appendix I). The medium (5 ml) was dispensed in each of the test tubes and autoclaved. The carbohydrate fermentation discs (Himedia®- DD006, DD004, DD005, DD003, DD006 and DD0012) were then dispensed into these test tubes. The bacterial inoculum *viz.*, 50 µl of 48 h. old liquid culture of the test isolate in CPG broth was added into each test tube. The un-inoculated tubes containing sterile Hayward's basal medium with carbohydrate fermentation discs served as control. The tubes were incubated at room temperature and observed for colour change for 21 days. The change in the colour of the medium from green to yellow indicated utilisation of the sugar.

3.7 ANALYSIS OF SOIL PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

The physical, chemical and biological properties of rhizosphere soil healthy and diseased plants of the eight locations were estimated. The physical properties analyzed include soil temperature, soil texture, water holding capacity and bulk density. The chemical properties estimated were soil pH, organic carbon, available P, available K and Ca contents and micronutrients like Fe and Mn content. The soil biological properties represented by population of rhizosphere microflora which includes fungi, bacteria, actinomycetes and fluorescent pseudomonads were estimated by serial dilution and plating.

3.7.1 Physical properties of soil

3.7.1.1 Estimation of soil temperature

The soil temperature was recorded *in-situ* at the time of soil sampling from all the eight locations. The rhizosphere soil temperature at 15 cm depth was measured for healthy and diseased plants of all locations.

3.7.1.2 Determination of soil texture

The soil texture was determined by 'soil-texture-by-feel method' as described by Thien (1979). The flow chart for the determination of soil texture by feel is given in Fig. 3.1.

3.7.1.3 Determination of soil water holding capacity and bulk density

The water holding capacity and bulk density of rhizosphere soil of healthy and diseased plants were determined using Keen-Raczkowski brass cup method (Piper, 1942).

Procedure

1. A filter paper was placed on the perforated bottom of the Keen-Raczkowski box and weighed.

2. The soil was then packed within the box by adding small quantities at a time and taping the box each after each addition to ensure even distribution. This was continued till the box was nearly full.

3. The surface was leveled with the straight edge of spatula when the box was completely full and the weight of the box along with the soil was recorded.

4. The box was then placed in a small tray containing water and was left over-night. Next day the box containing saturated soil was removed from the tray and weighed.

5. The expanded wet soil from the box was removed using a sharp scalpel and transferred to a pre-weighed petri dish. The weight of the petri dish along with the wet soil was taken. The weight of the box with the residual wet soil was also recorded.

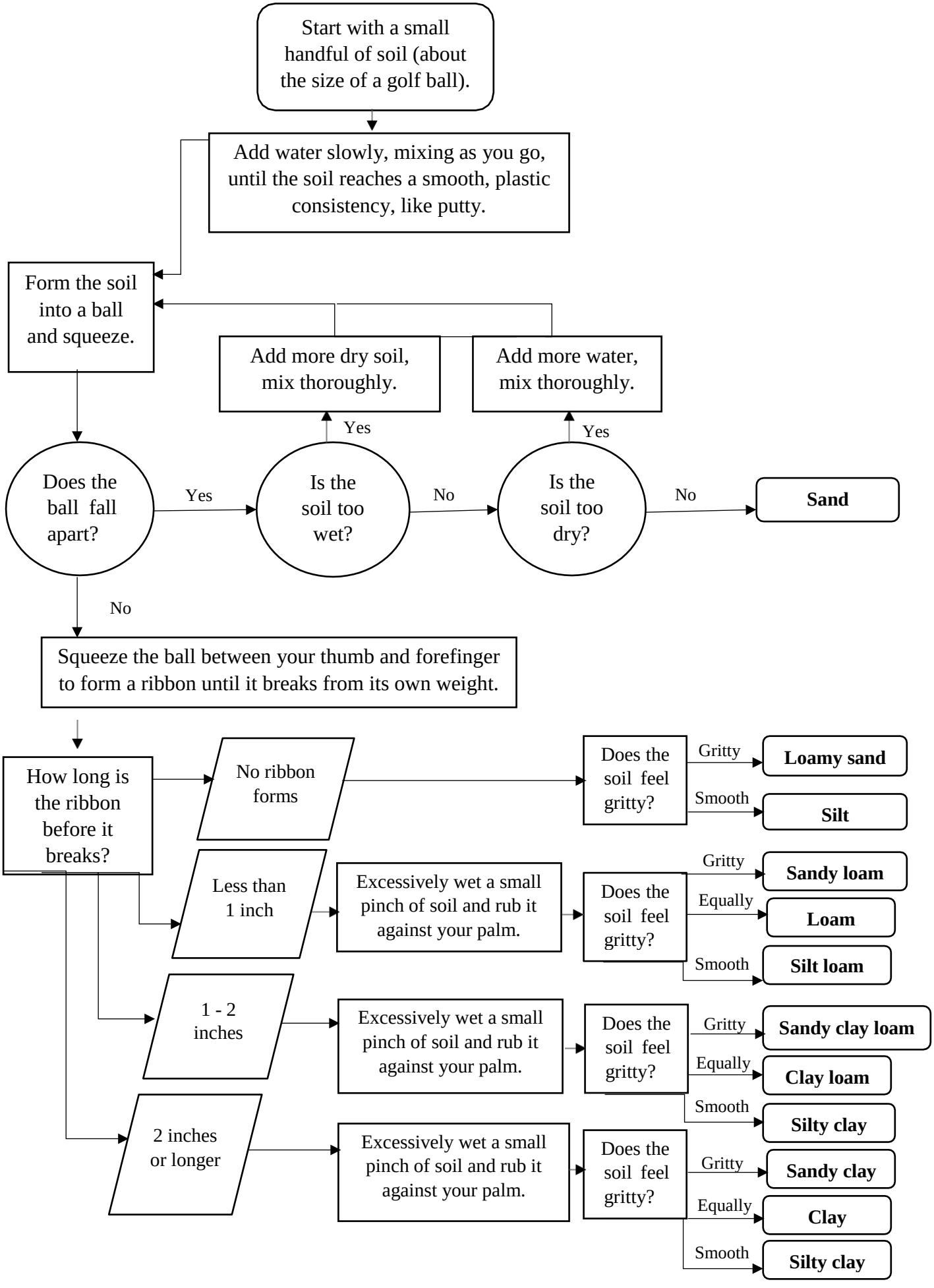


Fig. 3.1 Flow chart for determination of soil texture by feel method

6. These were dried in an oven at 105° C till the weight was constant. Finally the weight of the dried soil was recorded. From the readings, the water holding capacity (WHC) and core bulk density was calculated using the given formula.

$$\text{WHC (\%)} = \frac{\text{Weight of the box+ saturated soil} - \text{Weight of the box+ dry soil}}{\text{Weight of the box+ dry soil} - \text{Weight of the box+ filter paper}} \times 100$$

$$\text{Bulk density (g/cm}^3\text{)} = \frac{\text{Weight of the soil (g)}}{\text{Volume of soil with pore space (cm}^3\text{)}}$$

$$= \frac{\text{Weight of the box+ dry soil - Weight of the box+ filter paper}}{\text{Volume of the box}}$$

3.7.2 Chemical properties of soil

Chemical analysis of rhizosphere soil of healthy and diseased plant samples were done using 0.5 mm sieved soil. Chemical characteristics of the soil *viz.*, pH, organic carbon, available P, K, Ca and micronutrients like Fe and Mn were analyzed as per the protocols given in Table 3.8.

Table 3.8 Methods used for estimation of chemical properties of soil

Sl No.	Chemical Properties	Methods		Reference
		Extraction	Estimation	
1.	pH	1:2.5 soil water suspension	Potentiometry	Jackson (1958)
2.	Organic carbon	Wet digestion		Walkely and Black (1934)
3.	Available P	Bray No.1	Colorimetry	Bray and Kurtz (1945)
4.	Available K	1N NH_4OAc	Flame Photometry	Jackson (1958)

5.	Available Ca	1N NH_4OAc	ICP OES (Model: Optima 8x00 series)	Jackson (1958)
6.	Available micronutrients (Fe, Mn)	0.1 N HCl	ICP OES (Model: Optima 8x00 series)	Sims and Johnson (1991)

3.7.3 Quantitative estimation of rhizosphere soil microflora

The quantitative estimation of soil microflora in rhizosphere soil of healthy and diseased plants were carried out by serial dilution and plating as described by Johnson and Curl (1992). For this, 10 g of soil sample was added to 90 ml of sterile water taken in 250 ml conical flask and shaken for 30 min. in orbital shaker (150 rpm). Pipette out one millilitre of soil suspension (10^{-1}) to nine millilitre of sterile water taken in test tube to get a dilution of 10^{-2}. Likewise, serial dilutions were prepared up to 10^{-7}. The dilutions and media used for the enumeration of different groups of microorganisms are given in Table 3.9.

Table 3.9 Details of dilutions and media used for enumeration of rhizosphere microflora

Sl No.	**Organism**	**Dilution**	**Medium**	**Period of incubation**
1.	Fungi	10^{-3}	Martin's Rosebengal streptomycin Agar	24 h.
2.	Bacteria	10^{-6}	Nutrient Agar	48 h.
3.	Actinomycetes	10^{-5}	Ken Knight's Agar	Seven days
4.	Fluorescent pseudomonads	10^{-7}	King's B Agar	48 h.

3.8 STATISTICAL ANALYSIS

Physico-chemical and biological properties as well as population of *R. solanacearum* in the rhizosphere soil of healthy and diseased plants were compared using paired samples t-test. Further, the correlation between the soil physico-chemical and biological properties and per cent disease incidence (PDI) as well as pathogen population in rhizosphere soil were analyzed. The correlation between population of pathogen in soil and PDI was also analyzed. To find out the soil parameters determining the disease incidence and built up of pathogen population in soil as well as their per cent contribution, step-up multiple regression analysis was performed. All the statistical analyses were carried out in SPSS v.16.

4. Results

4. RESULTS

Investigations on 'Characterisation of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* infecting solanaceous vegetables in relation to physico-chemical and biological properties of soil' was undertaken at the Department of Plant Pathology, College of Horticulture, Vellanikkara. The study comprised of characterisation of *R. solanacearum* isolates collected from four different agro ecological units (AEUs) of Kerala and analysis of soil properties influencing the disease. The results of the study are presented below.

4.1 SURVEY ON BACTERIAL WILT OF SOLANACEOUS CROPS

A purposive sampling survey was conducted in four AEUs of Kerala *viz.* North central laterite (NCP), Marayur hills (MH), Southern laterite (SL) and Palakkad central plains (PCP). Two locations from each of these AEUs were surveyed during March to November 2018 and per cent disease incidence of bacterial wilt was recorded. The highest PDI of 88 was observed in the location NCL 1 (Pudukkad) where the crop was brinjal. It was followed by SL 2 (Venganoor) and SL 1 (Pravachambalam) where the PDI recorded were 72.3 and 86.7 respectively. The bacterial wilt incidence was less than 40 per cent in the remaining locations and the lowest PDI (20) was observed in PCP 1 (Vithanassery). The crop grown at the time of survey was potato in Marayur hills, tomato in NCL 2 and brinjal in all other locations (Plate 4.1). The details of the survey on bacterial wilt of solanaceous crops are given in Table 4.1.

4.2 ISOLATION AND PATHOGENICITY TEST

4.2.1 Isolation of the pathogen

The bacterium associated with the wilted plant samples collected during the survey was isolated on TZC agar. The bacterial ooze collected from the basal stem portion of the plant was used in the case of tomato and brinjal, while the ooze exuding from symptomatic tubers were used in the case of potato. Typical creamy white colonies with pink or red center were observed on TZC agar suggesting the presence of *R. solanacearum*.

Table 4.1 Survey on bacterial wilt of solanaceous crops

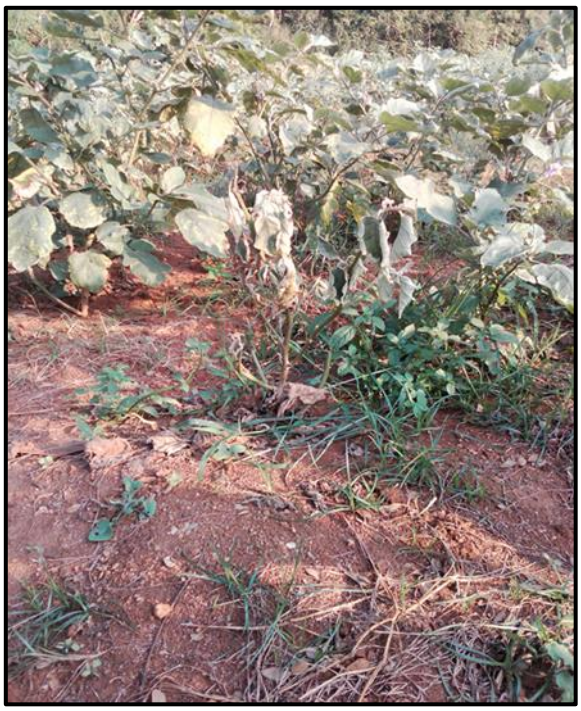
Sl No.	Location	GPS coordinates	Crops	PDI	Pathogen population (x 10^4cfu g^{-1} of soil)	
					Diseased	Healthy
1.	NCL 1 (Pudukkad)	10° 25´ 15.87´´N 76° 14´35.102´´E	Brinjal	88.00	102.00	31.33
2.	NCL 2 (Vellanikkara)	10° 33´ 6.037´´N 76° 17´ 5.413´´E	Tomato	36.70	31.33	22.33
3.	MH 1 (Perumalai)	10° 12´ 19.541´´N 77° 11´ 26.188´´E	Potato	35.70	29.66	22.66
4.	MH 2 (Kanthaloor)	10° 12´ 14.998´´N 77° 11´ 26.073´´E	Potato	25.80	25.66	20.66
5.	SL 1 (Pravachambalam)	8° 23´ 47.251´´N 77° 0´ 14.356´´E	Brinjal	72.30	126.33	57.66
6.	SL 2 (Venganoor)	8° 24´ 16.279´´N 77° 0´ 57.344´´E	Brinjal	86.70	137.66	98.33
7.	PCP 1 (Vithanassery)	10° 36´ 22.172´´N 76° 35´ 36.992´´E	Brinjal	20.00	39.00	27.33
8.	PCP 2 (Vithanassery)	10° 36´ 28.098´´N 76° 38´ 3.155´´E	Brinjal	41.60	43.00	36.66

NCL – Northern central laterite

MH – Marayur hills

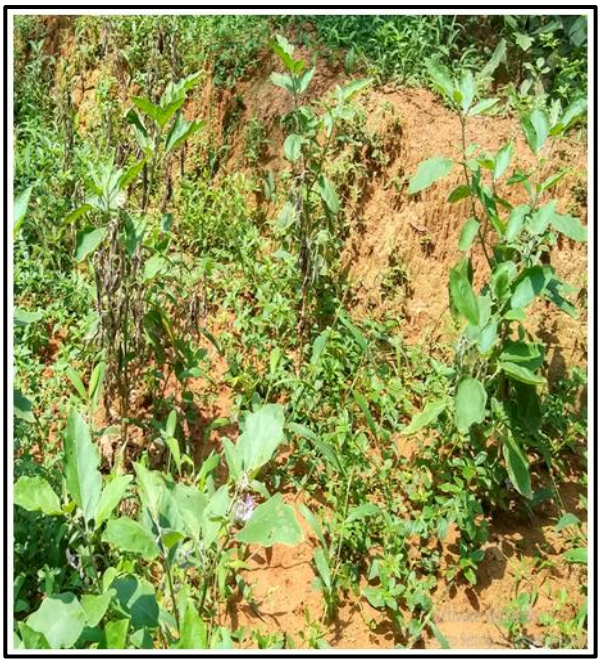
SL – Southern laterite

PCP – Palakkad central plains



(a) NCL 1

(b) NCL 2



(c) SL 1

(d) SL 2

Plate 4.1 Survey on bacterial wilt in solanaceous crops

(e) MH 1

(f) MH 2

(g) PCP 1

(h) PCP 2

Plate 4.1 Survey on bacterial wilt in solanaceous crops

4.2.2 PATHOGENICITY TEST

4.2.2 Pathogenicity test

Inoculation of fresh bacterial ooze collected from wilted brinjal, tomato and potato to susceptible varieties of respective host plants produced characteristic wilt symptoms. In the case of brinjal and tomato, wilting symptoms were observed within nine to ten days after inoculation whereas in potato wilting was seen within 15 days after inoculation. The presence of the bacterium was confirmed by ooze test and the pathogen was re-isolated from the wilted plants. The colonies formed on TZC agar were identical to those mentioned in section 4.2.1, thus confirming the pathogenicity of the bacterium.

4.2.3 Purification and maintenance of isolates

The colonies appearing creamy white with pink or red center on TZC agar were selected and purified by repeated streaking on casaminoacid peptone glucose (CPG) agar. Three to five colonies exhibiting typical colony characteristics were picked and suspended in sterile distilled water in screw-capped glass vials and stored at room temperature. The identity and viability of the isolates were tested on TZC agar once in two months and were found to produce pink or red centered, creamy white colonies indicating viability of the pathogen.

4.3 QUANTIFICATION OF POPULATION OF PATHOGEN IN RHIZOSPHERE

The population of *R. solanacearum* in rhizosphere soil of diseased and healthy plants was estimated using selective medium South Africa (SMSA). The typical creamy white colonies with red center characteristic of the pathogen were observed without any microbial contamination (Plate 4.2). In general, the population was higher in rhizosphere of diseased plants compared to that of healthy (Table 4.1). The population ranged from 20.66 x 10^4 cfu g^{-1} soil to 98.33 x 10^4 cfu g^{-1} soil in rhizosphere of healthy plants and from 23.66 x 10^4 cfu g^{-1} to 137.66 x 10^4 cfu g^{-1} soil of diseased plants. The results of the paired sample t-test revealed significant difference in pathogen population between the rhizosphere soil of diseased and healthy plants. The pathogen was most abundant in SL 2 (137.66 x 10^4 cfu g^{-1} soil) followed by SL 1 (126.33 x 10^4 cfu g^{-1} soil) and NCL 1 (102

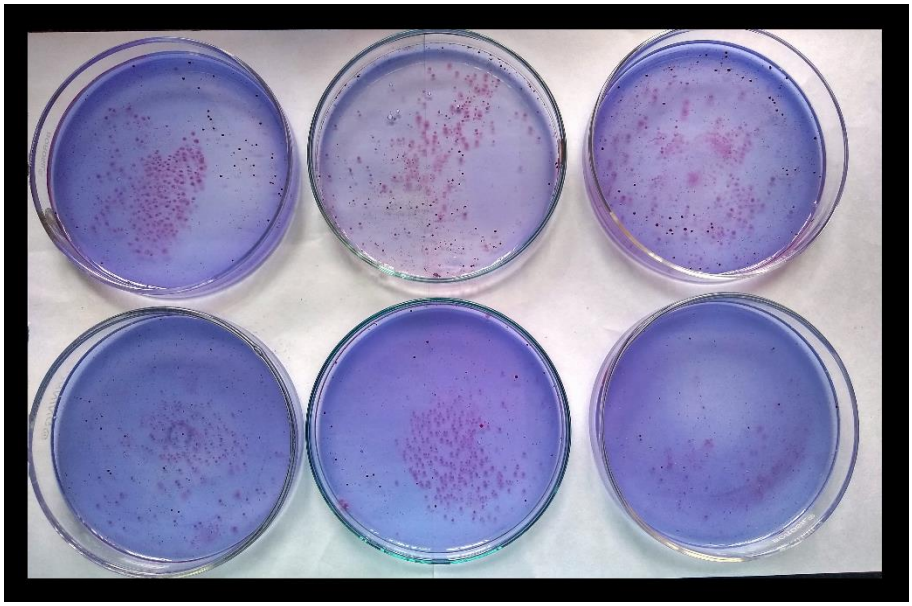


Plate 4.2 Colonies of *Ralstonia solanacearum* on SMSA -E

x 10^4 cfu g^{-1} soil) whereas the lowest population of the bacterium was recorded in MH 2 (20.66 x 10^4 cfu g^{-1} soil). A significant positive correlation (r = 0.921) was observed between PDI and population of the pathogen in soil.

4.4 CHARACTERISATION OF THE PATHOGEN

4.4.1 Cultural characterisation

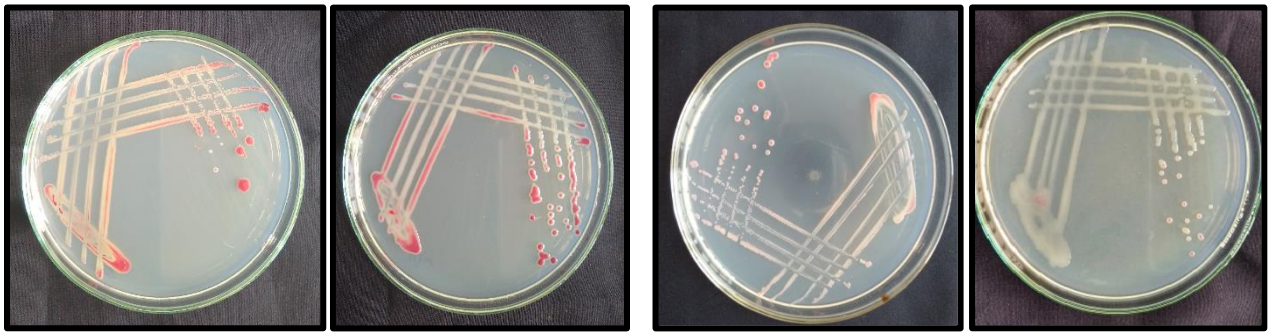
The colony characteristics of all the isolates of the pathogen were similar though slight variations were observed (Table 4.2). The size of the colonies varied from 1.5 mm to 5.5 mm. All the isolates produced circular colonies with entire margin. Colonies were slightly raised in the case of all the isolates except those from PCP 1 and PCP 2 which were flat in elevation. The colonies of isolates from Northern central laterite were creamy white with light red or pink center. The NCL 2 isolate had moderate fluidity compared to NCL 1. The isolates of pathogen from Marayur hills formed light pink centered colonies and fluidity of colonies was high in MH 2 while moderate in MH 1. The isolates collected from Southern laterite produced circular creamy white colonies with pink center while those from Palakkad central plains were dark red coloured with a dirty white outline and high fluidity (Plate 4.3).

4.4.2 Morphological characterisation

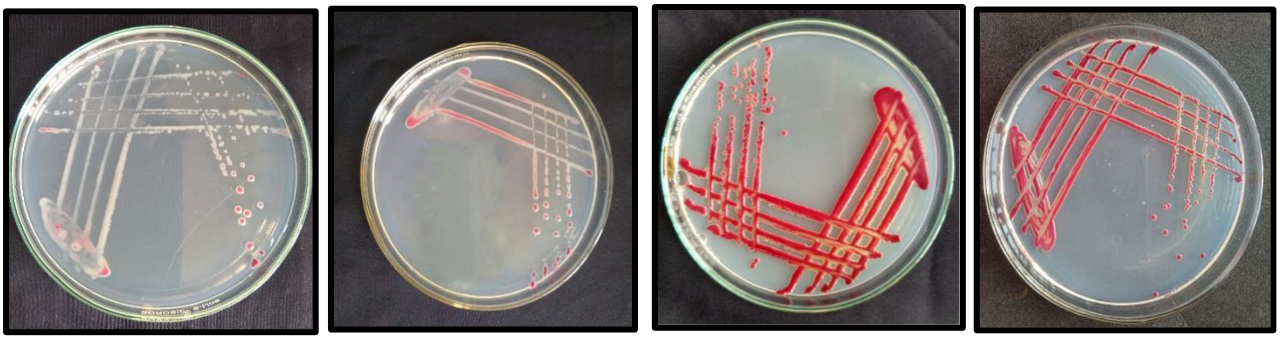
Morphological characterisation was carried out using Gram's staining and scanning electron microscopy (SEM). All the isolates were negative to Gram's reaction. The morphology of the bacterial cells were observed using scanning electron microscope. The cells were initially fixed to preserve the structure followed by dehydration and drying. Completely dried cells were sputter-coated with gold and viewed under SEM. The working distance of the electron beam and magnification were optimized by trial and error method in order to get high resolution images. High quality images with the best resolution were obtained at 17.85 mm working distance with a magnification of 16.2 kX (Plate 4.4a) and also at a working distance of 8.98 mm with a magnification of 44.9 kX (Plate 4.4b). Typical rod shaped cells of *R. solanacearum* of size 0.3-0.5 µm x 1.2-1.7 µm were observed.

Table 4.2 Colony characteristics of the isolates

Isolate	Size	Pigmentation	Margin	Form	Elevation	Fluidity
NCL 1	1.5 mm -3.5 mm	Creamy white with light red center	Entire	Circular	Slightly raised	Low
NCL 2	3.5 mm -5.5 mm	Creamy white with dark pink center	Entire	Circular	Slightly raised	Moderate
MH 1	1.5 mm -3.0 mm	Creamy white with light pink center	Entire	Circular	Slightly raised	Moderate
MH 2	1.5 mm -2.5 mm	Creamy white with light pink center	Entire	Circular	Slightly raised	High
SL 1	3.5 mm -5.5 mm	Creamy white with pink center	Entire	Circular	Slightly raised	Moderate
SL 2	3.5 mm -4.5 mm	Creamy white with pink center	Entire	Circular	Slightly raised	Moderate
PCP 1	2.5 mm -3.5 mm	Dark-red colored colonies with white outline	Entire	Circular	Flat	High
PCP 2	2.0 mm -3.5 mm	Dark-red colored colonies with white outline	Entire	Circular	Flat	High

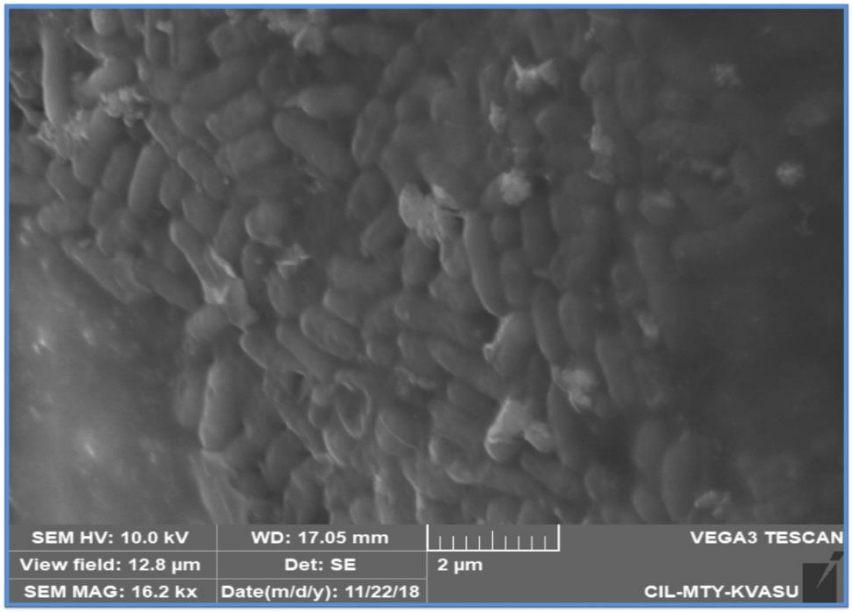


(a) NCL 1 **(b) NCL 2** **(c) MH 1** **(d) MH 2**

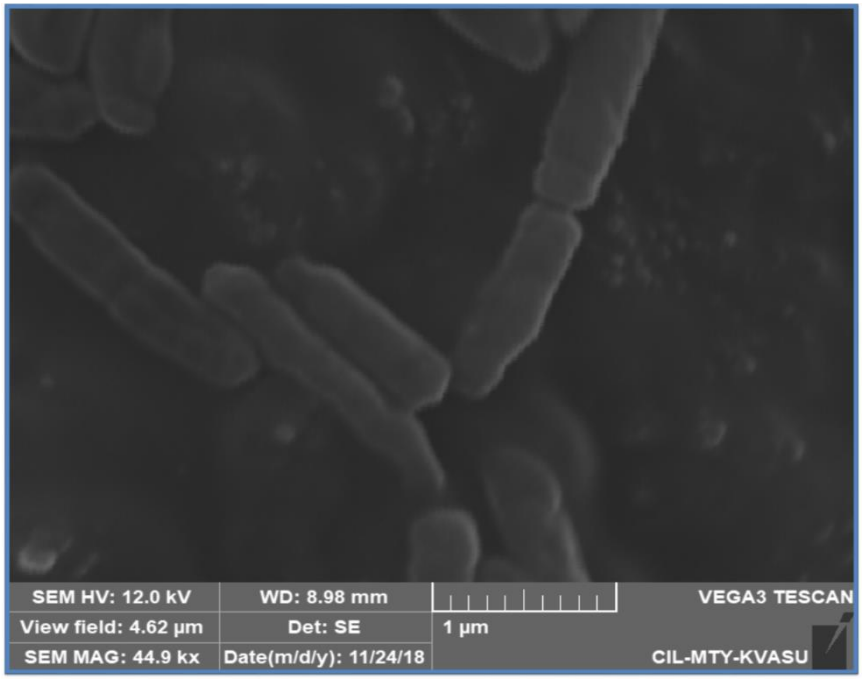


(e) SL 1 **(f) SL 2** **(g) PCP 1** **(h) PCP 2**

Plate 4.3 Isolation of *Ralstonia solanacearum* from different regions of Kerala



(a) *Ralstonia solanacearum* cells at 16.2 kX magnification



(b) *Ralstonia solanacearum* cells at 44.9 kX magnification

Plate 4.4 Scanning electron micrograph of *Ralstonia solanacearum*

4.4.3 Molecular characterisation

4.4.3.1 Isolation of genomic DNA

The total genomic DNA of the eight bacterial isolates collected were isolated using the protocol mentioned in the section 3.4.3.1. The precipitate of DNA visible as white strands was centrifuged and the DNA pellets were obtained which were then washed in ethanol, air dried and dissolved in distilled water for further studies.

4.4.3.2 Qualitative analysis of DNA using agarose gel electrophoresis

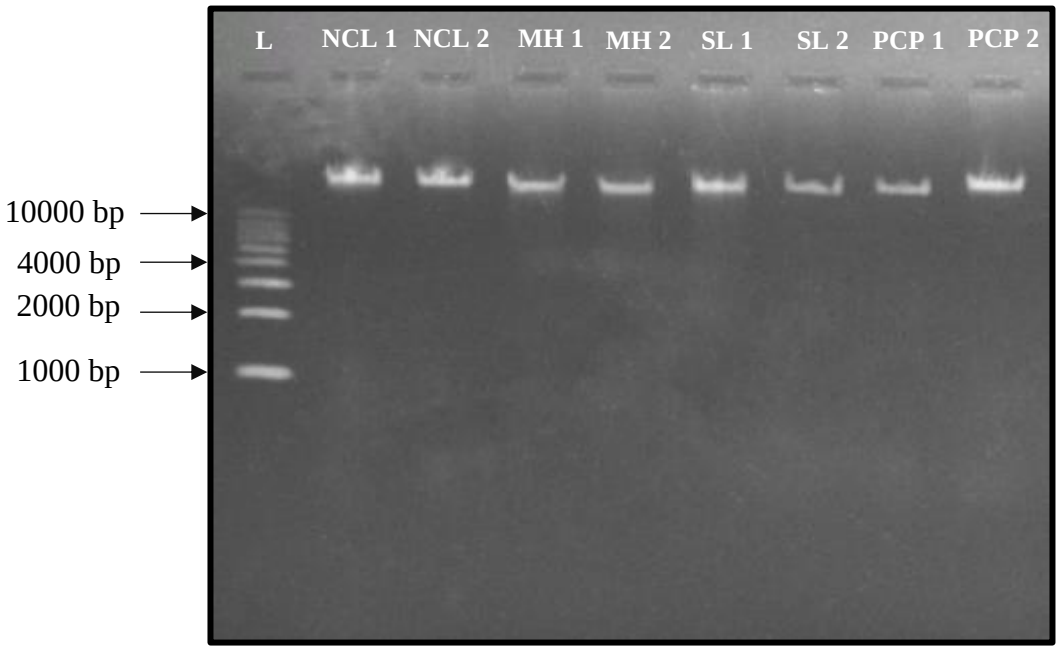
Quality of the DNA isolated was assessed by agarose gel electrophoresis. Intact bands of DNA without much contamination (Plate 4.5) visualized in gel documentation system indicated high quality and suitability for molecular characterisation.

4.4.3.3 Quantification and purity analysis of DNA

The quantity and purity of DNA samples were assessed using Nanodrop spectrophotometer and the data are presented in the Table 4.4. The absorbance ratio of the bacterial DNA obtained from the isolates ranged from 1.78 to 1.99 and the yield of DNA ranged from 891.47 ng μl^{-1} to 235.73 ng μl^{-1}. The absorbance values of bacterial DNA of all isolates were in between 1.8 to 2.0, which indicated the purity of DNA without any RNA or protein contamination.

4.4.3.4 PCR amplification of 16S rRNA

The 16S rRNA gene contained in the genomic DNA was amplified using the set of universal primers 8F and 1522R. The best concentration of DNA template and annealing temperature were standardized based on the quality of band obtained during gel electrophoresis. A concentration of 30 ng μl^{-1} of DNA template and an annealing temperature of 55ºC were selected and amplification was carried out.



Ladder – 1 Kb

Plate 4.5 Genomic DNA of *R. solanacearum* isolates

Table 4.3 Quality and quantity of DNA of *R. solanacearum* isolates

Isolate	A $_{260/280}$	Quantity of DNA (ng/µl)
NCL 1	1.99	240.42
NCL 2	1.83	464.26
MH 1	1.95	891.47
MH 2	1.78	292.84
SL 1	1.89	298.73
SL 2	1.81	615.49
PCP 1	1.83	235.73
PCP 2	1.98	375.80

4.4.3.4.1 Analysis of PCR products by gel electrophoresis

The amplicons were analyzed in agarose gel (1%) in TAE buffer (1X). The PCR products (5 µl) were loaded in consecutive wells with 1 Kbp ladder in the first well to determine the size of the product. The amplicon size of the PCR products was 1.5 Kbp and all amplicons of all the isolates were of same size (Plate 4.6).

4.4.3.4.2 Sequencing of 16S rDNA gene

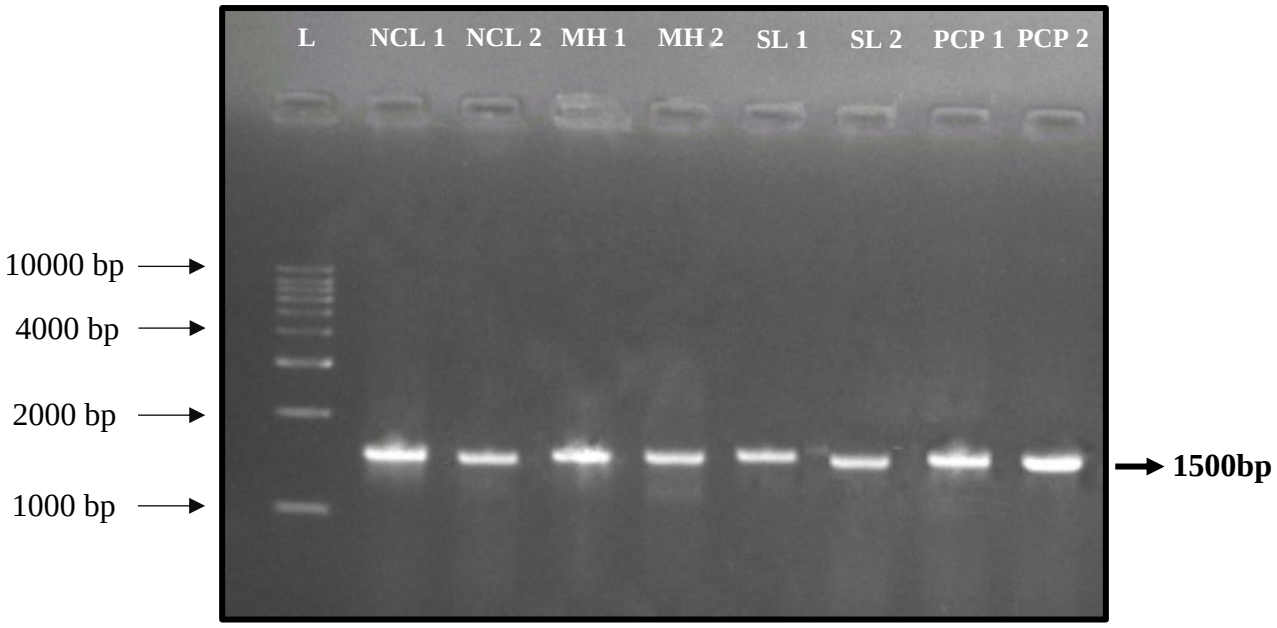
The PCR products of the eight isolates were sent to Agri Genome, Kochi and Rajiv Gandhi Center for Biotechnology for purification and sequencing. The nucleotide sequence data obtained are given in Appendix III.

4.4.3.5 In-silico analysis 16S rRNA sequences

Sequence analysis was carried out using nucleotide basic alignment search tool (BLASTn) to compare the sequences of eight isolates with those available in NCBI database and identify those sequences that resemble with the query sequence above a certain threshold. All the eight query sequences showed significant similarity to the reported sequences of 16S rDNA gene of *Ralstonia solanacearum* in the NCBI database (Table 4.4 to 4.11).

The sequence of the isolate NCL 1 showed 99.84 per cent identity with *R. solanacearum* strain RS481 from China with maximum query cover of 100 per cent. The sequence also showed homology with the *R. solanacearum* strains RS480, RS479 from China. Whereas, the sequence of NCL 2 showed 100 per cent identity with *R. solanacearum* strain S1 of tomato with query coverage of 100 per cent and an expected value of zero. The isolate also showed 100 per cent identity with all the sequences producing significant alignments with an expected value of zero and a total score of 2333.

The nucleotide blast of sequence of the isolate MH 1 showed 100 per cent identity with *R. solanacearum* strain BGR26 from China with a maximum query cover of 100 per cent and an expected value of zero. The sequence also showed 99.39 per cent identity with *R. solanacearum* strain 08BF31TG with maximum query cover of 100 per cent. The



Ladder – 1 Kb

Plate 4.6 PCR gel profile of 16S rDNA of *R. solanacearum* isolates

sequence homology analysis of the isolate MH 2 revealed a per cent identity of 100 with *R. solanacearum* strain CLYM2 with a query cover of 100 per cent. Also, *R. solanacearum* strains LEYM2, STYM2 and SMYM1 from China exhibited 99.79 per cent identity with the given query sequence.

The sequence of the isolate SL 1 showed 99.59 per cent identity with *R. solanacearum* RS380 of China with a maximum query coverage of 100 per cent. The isolate also exhibited similar per cent identity with *R. solanacearum* strains RS379, RS378, RS377 *etc*. *In-silico* analysis of the sequence of isolate SL 2 revealed 100 per cent identity to *R. solanacearum* strain JN-1 [MK696210.1] of China with a query cover of 98 per cent. The isolate SL 2 also showed 100 per cent identity to *R. solanacearum* strains YC-2, YC-1, YZ-2 *etc*. The significant alignments had the same per cent identity of 100 and query cover of 98 per cent.

The BLASTn output of the sequence of the isolate PCP 1 displayed 100 per cent identity with *R. solanacearum* strain JN-1 of China with a query cover of 100 per cent. Also, *R. solanacearum* strains 9-2, ICMP 20038, Tom 1, *etc*. showed 100 per cent identity with the query sequence. Homology analysis of the sequence of isolate PCP 2 revealed a per cent identity of 99.92 with the *R. solanacearum* strain RS379 with a maximum query cover of 100 per cent. The isolate also showed similar per cent identity with *R. solanacearum* strains RS378 RS377, RS376 *etc*.

4.4.3.6 Phylogenetic analysis

Phylogenetic analysis was carried out for understanding the relationship of the isolates collected during the present study with other isolates of *R. solanacearum* reported from India and elsewhere. The 16S rDNA nucleotide sequence of the eight isolates of different locations were aligned with 21 other sequences of *R. solanacearum* 16S rDNA gene retrieved from NCBI database. The sequences were aligned using the Clustal W software and a neighbor joining phylogenetic tree was constructed employing the MEGA X software (Fig.4.1).

Table 4.4 *In-silico* analysis of *Ralstonia solanacearum* isolate NCL 1

Species	Accession number	Total score	Query cover	Per cent identity	E - value
Ralstonia solanacearum strain RS 481 16S ribosomal RNA partial sequence	MK 125273.1	2300	100%	99.84%	0.0
Ralstonia solanacearum strain RS 480 16S ribosomal RNA partial sequence	MK 125272.1	2300	100%	99.84%	0.0
Ralstonia solanacearum strain RS 479 16S ribosomal RNA partial sequence	MK 125271.1	2300	100%	99.84%	0.0
Ralstonia solanacearum strain RS 478 16S ribosomal RNA partial sequence	MK 125273.1	2300	100%	99.84%	0.0
Ralstonia solanacearum strain JN 1 16S ribosomal RNA partial sequence	MK 696210.1	2300	100%	99.84%	0.0
Ralstonia solanacearum strain YC 2 16S ribosomal RNA partial sequence	MK 696209.1	2300	100%	99.84%	0.0
Ralstonia solanacearum strain YC 1 16S ribosomal RNA partial sequence	MK 696208.1	2300	100%	99.84%	0.0

Table 4.5 *In-silico* analysis of *Ralstonia solanacearum* isolate NCL 2

Species	Accession number	Total score	Query cover	Per cent identity	E value
Ralstonia solanacearum strain S1 16S ribosomal RNA partial sequence	MK 990030.1	2333	98%	100%	0.0
Ralstonia solanacearum strain RS 494 16S ribosomal RNA partial sequence	MK 125286.1	2333	98%	100%	0.0
Ralstonia solanacearum strain RS 493 16S ribosomal RNA partial sequence	MK 125285.1	2333	98%	100%	0.0
Ralstonia solanacearum strain RS 492 16S ribosomal RNA partial sequence	MK 125284.1	2333	98%	100%	0.0
Ralstonia solanacearum strain RS 491 16S ribosomal RNA partial sequence	MK 125283.1	2333	98%	100%	0.0
Ralstonia solanacearum strain RS 490 16S ribosomal RNA partial sequence	MK 125282.1	2333	98%	100%	0.0
Ralstonia solanacearum strain RS 489 16S ribosomal RNA partial sequence	MK 125281.1	2333	98%	100%	0.0

Table 4.6 *In-silico* analysis of *Ralstonia solanacearum* isolate MH 1

Species	Accession number	Total score	Query cover	Per cent identity	E value
Ralstonia solanacearum strain BGR 26 16S ribosomal RNA partial sequence	KC 789781.1	2416	100%	100%	0.0
Ralstonia solanacearum strain 08BF31TG 16S ribosomal RNA partial sequence	KX 146476.1	2372	100%	99.39%	0.0
Ralstonia solanacearum strain B 4 16S ribosomal RNA partial sequence	KT 359571.1	2368	99%	99.39%	0.0
Ralstonia solanacearum strain SC 161 16S ribosomal RNA partial sequence	MH 256546.1	2366	100%	99.31%	0.0
Ralstonia solanacearum strain BTHNGU 38 16S ribosomal RNA partial sequence	KT 010359.1	2361	100%	99.31%	0.0
Ralstonia solanacearum strain B 5 ribosomal RNA partial sequence	KT 359576.1	2353	100%	99.28%	0.0
Ralstonia solanacearum strain UW 386 16S ribosomal RNA partial sequence	CP 039576.1	2350	100%	99.28%	0.0

Table 4.7 *In-silico* analysis of *Ralstonia solanacearum* isolate MH 2

Species	Accession number	Total score	Query cover	Per cent identity	E - value
Ralstonia solanacearum strain CLY M2 16S ribosomal RNA partial sequence	KC 620463.1	2660	100%	100%	0.0
Ralstonia solanacearum strain CLY M1 16S ribosomal RNA partial sequence	KX 620472.1	2654	100%	99.79%	0.0
Ralstonia solanacearum strain LEY M2 16S ribosomal RNA partial sequence	KC 620471.1	2643	99%	99.79%	0.0
Ralstonia solanacearum strain STY M2 16S ribosomal RNA partial sequence	KC 620470.1	2643	100%	99.79%	0.0
Ralstonia solanacearum strain SMY M1 16S ribosomal RNA partial sequence	KC 620467.1	2643	100%	99.79%	0.0
Ralstonia solanacearum strain MPY M1 ribosomal RNA partial sequence	KC 620465.1	2643	100%	99.72%	0.0
Ralstonia solanacearum strain STY M1 16S ribosomal RNA partial sequence	KC 620469.1	2643	100%	99.72%	0.0

Table 4.8 *In-silico* analysis of *Ralstonia solanacearum* isolate SL 1

Species	Accession number	Total score	Query cover	Per cent identity	E - value
Ralstonia solanacearum strain RS 380 16S ribosomal RNA partial sequence	KY 594786.1	2222	100%	99.59%	0.0
Ralstonia solanacearum strain RS 379 16S ribosomal RNA partial sequence	KX 584785.1	2222	100%	99.59%	0.0
Ralstonia solanacearum strain RS 378 16S ribosomal RNA partial sequence	KC 594784.1	2222	99%	99.59%	0.0
Ralstonia solanacearum strain RS 377 16S ribosomal RNA partial sequence	KC 594783.1	2222	100%	99.59%	0.0
Ralstonia solanacearum strain RS 376 16S ribosomal RNA partial sequence	KC 594782.1	2222	100%	99.59%	0.0
Ralstonia solanacearum strain RS 375 16S ribosomal RNA partial sequence	KC 594781.1	2222	100%	99.59%	0.0
Ralstonia solanacearum strain RS 374 16S ribosomal RNA partial sequence	KC 594780.1	2222	100%	99.59%	0.0

Table 4.9 *In-silico* analysis of *Ralstonia solanacearum* isolate SL 2

Species	Accession number	Total score	Query cover	Per cent identity	E - value
Ralstonia solanacearum strain JN 1 16S ribosomal RNA partial sequence	MK 696210.1	2597	100%	100%	0.0
Ralstonia solanacearum strain 9 - 2 16S ribosomal RNA partial sequence	KP 729638.1	2597	100%	100%	0.0
Ralstonia solanacearum strain ICMP 20038 16S ribosomal RNA partial sequence	KM 216391.1	2597	99%	100%	0.0
Ralstonia solanacearum strain Tom 1 16S ribosomal RNA partial sequence	KM 084995.1	2597	99%	100%	0.0
Ralstonia solanacearum strain YC 1 16S ribosomal RNA partial sequence	MK 696208.1	2597	99%	100%	0.0
Ralstonia solanacearum strain DIBER119 16S ribosomal RNA partial sequence	MG 266201.1	2597	99%	100%	0.0
Ralstonia solanacearum strain Tom 6 16S ribosomal RNA partial sequence	KM 085000.1	2597	99%	100%	0.0

Table 4.10 *In-silico* analysis of *Ralstonia solanacearum* isolate PCP 1

Species	Accession number	Total score	Query cover	Per cent identity	E - value
Ralstonia solanacearum strain JN 1 16S ribosomal RNA partial sequence	MK 696210.1	2333	98%	100%	0.0
Ralstonia solanacearum strain YC 2 16S ribosomal RNA partial sequence	MK 696209.1	2333	98%	100%	0.0
Ralstonia solanacearum strain YC 1 16S ribosomal RNA partial sequence	MK 696208.1	2333	98%	100%	0.0
Ralstonia solanacearum strain YZ 1 16S ribosomal RNA partial sequence	MK 696207.1	2333	98%	100%	0.0
Ralstonia solanacearum strain YZ 2 16S ribosomal RNA partial sequence	MK 696206.1	2333	98%	100%	0.0
Ralstonia solanacearum strain DIBER116 16S ribosomal RNA partial sequence	MG 266202.1	2333	98%	100%	0.0
Ralstonia solanacearum strain DIBER 118 16S ribosomal RNA partial sequence	MG 266203.1	2333	98%	100%	0.0

Table 4.11 *In-silico* analysis of *Ralstonia solanacearum* isolate PCP 2

Species	Accession number	Total score	Query cover	Per cent identity	E - value
Ralstonia solanacearum strain RS 379 16S ribosomal RNA partial sequence	KY 594785.1	2303	100%	99.92%	0.0
Ralstonia solanacearum strain RS 378 16S ribosomal RNA partial sequence	KY 594784.1	2303	100%	99.92%	0.0
Ralstonia solanacearum strain RS 377 16S ribosomal RNA partial sequence	KY 594783.1	2303	99%	99.92%	0.0
Ralstonia solanacearum strain RS 376 16S ribosomal RNA partial sequence	KY 594782.1	2303	100%	99.92%	0.0
Ralstonia solanacearum strain RS 375 16S ribosomal RNA partial sequence	KY 594781.1	2303	100%	99.92%	0.0
Ralstonia solanacearum strain RS 374 16S ribosomal RNA partial sequence	KY 594780.1	2303	100%	99.92%	0.0
Ralstonia solanacearum strain RS 373 16S ribosomal RNA partial sequence	KY 594779.1	2303	100%	99.92%	0.0

The phylogenetic analysis of the nucleotide sequences of the isolates collected in the study, along with 21 other sequences in the database resulted in multiple groups (Fig.4.1). The phylogenetic tree branched into two major clusters and the isolates collected from NCL, PCP and SL were seen distributed within one cluster while those from MH in another cluster. Of these, the isolates NCL 1 and SL 2 were grouped together and these along with isolate SL 1 was seen arising from a common node supported with a bootstrap value of 88. These isolates were seen related to BRs- Kal [JQ359668.1] (West Bengal) and IHRTRS-20 [KJ546141.1] (Karnataka) as they shared a common lineage. The isolates PCP 1, PCP 2 and NCL 2 formed a separate sub-cluster within the same cluster. The isolates from PCP 1 and NCL 2 formed a common cluster with a bootstrap value of 100 indicating that they are closely related to each other. These isolates showed similarity to strain CaRs-Mep [JF523189.1] from India and strains YC 2 [MK696209.1], JN 1 [MK 696163.1] and S1 [990036.1] from China at varying degrees. The Maryur isolates, that is, MH 1 and MH 2 formed a sub-cluster within the second cluster. This clustering was supported by a bootstrap value of 100 indicating that they have maximum probability of obtaining the same clustering pattern. They shared a common lineage with strains GRs-Sik [JQ 3596651.1] from Sikkim and CRs-Avl [JQ359676.1] from Ambalavayal as they emerged from a common node in the phylogenetic tree. Thus the phylogenetic analysis of eight isolates collected from different agro ecological units of Kerala along with the other 21 strains revealed that they formed a monophyletic group indicating their origin from a common ancestor. However, the local isolates belonged to different sub-clusters within the main cluster which indicated that there is considerable genetic diversity among the isolates collected in the study in accordance with the agro ecological unit from which they were collected.

4.5 IDENTIFICATION OF RACES BY PATHOGENICITY ON DIFFERENTIAL HOSTS

The eight isolates of *R. solanacearum* collected from four different agro ecological units were inoculated into differential host plants *viz*., tomato, brinjal, chilli, banana, potato, ginger and mulberry.

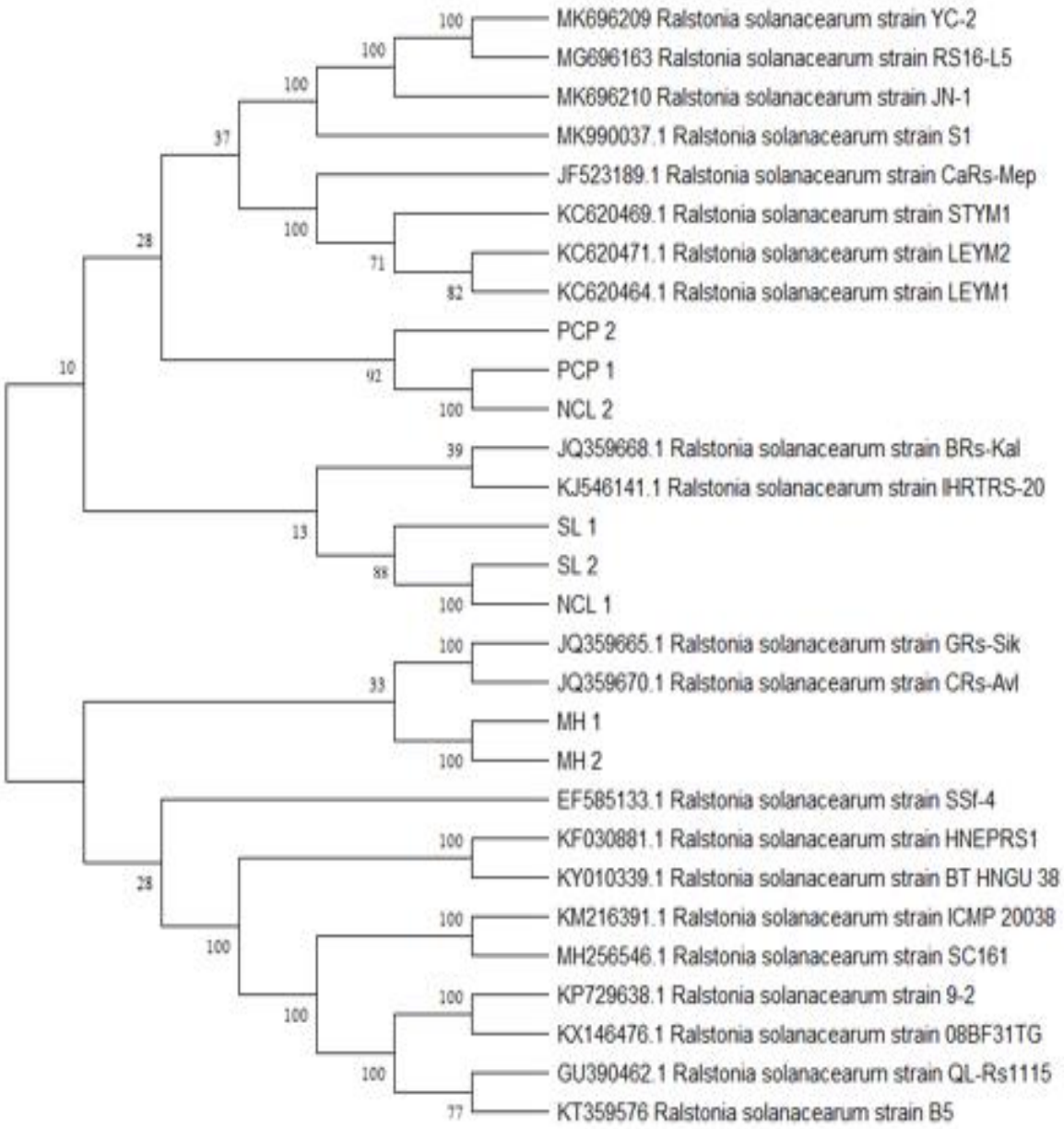


Fig. 4.1 Neighbour-joining tree representing the phylogenetic relationship of *Ralstonia solanacearum* isolates from Kerala
(Numbers are percentage support of branching based on bootstrap analysis, 1000 replicates).

The isolates *viz.*, NCL 1, NCL 2, SL 1, SL 2, PCP 1 and PCP 2 collected from wilted samples of brinjal and tomato induced wilting symptoms on tomato, brinjal and chilli within seven to 16 days after inoculation. However, these isolates failed to produce symptoms on potato. The isolates MH 1 and MH 2 collected from wilted samples of potato produced wilting symptoms on potato within 10 to 15 days after inoculation. These isolates were not able to infect other solanaceous host plants. All the collected isolates did not produce any symptoms on other differential hosts like banana, mulberry and ginger (Plate 4.7- 4.13). So based on infectivity on differential hosts, the isolates NCL 1,NCL 2, SL 1, SL 2, PCP 1, PCP 2 were designated as race 1 and the isolates MH 1 and MH 2 were classified as race 3 (Table 4.12).

4.6 IDENTIFICATION OF BIOVARS BY CARBOHYDRATE UTILISATION TEST

The different isolates of *R. solanacearum* collected from four different agro ecological units differed in their reaction to utilise hexose alcohols and disaccharides (Table 4.17, Plate 4.14 - 4.15) and in time required for the utilisation of these carbohydrates (Table 4.13 - 4.16). The isolate NCL 1 utilised all carbohydrates within four days whereas NCL 2 took seven days for the complete utilisation of carbohydrates. So the isolates NCL 1 and NCL 2 belonged to biovar III. The isolates of Marayur hills were much slower in the utilisation of sugars and hexose alcohols. Both the isolates *viz.*, MH 1 and MH 2 took seven days to utilise the disaccharides. Even after 14 days, the isolates could not oxidize various hexose alcohols and hence they were designated as biovar II. Among the isolates of Southern laterite, SL 2 utilised all the carbohydrates within three days while SL 1 took four days for complete oxidation of carbohydrates. Thus they were grouped into biovar III. Both the isolates of PCP were able to utilise all the disaccharides and hexose alcohols except dulcitol within three days. Even after 14 days, both PCP 1 and PCP 2 were not able to utilise dulcitol, hence they were identified as biovar IIIA.

Table 4.12 Identification of races of *R. solanacearum* by differential host technique

Strains	Differential hosts							Race
	Tomato	Brinjal	Chilli	Banana	Potato	Ginger	Mulberry	
NCL 1	Wilted	Wilted	Wilted	NS	NS	NS	NS	1
NCL 2	Wilted	Wilted	Wilted	NS	NS	NS	NS	1
MH 1	NS	NS	NS	NS	Wilted	NS	NS	3
MH 2	NS	NS	NS	NS	Wilted	NS	NS	3
SL 1	Wilted	Wilted	Wilted	NS	NS	NS	NS	1
SL 2	Wilted	Wilted	Wilted	NS	NS	NS	NS	1
PCP 1	Wilted	Wilted	Wilted	NS	NS	NS	NS	1
PCP 2	Wilted	Wilted	Wilted	NS	NS	NS	NS	1

NCL – Northern central laterite

MH – Maryur hills

SL – Southern laterite

PCP – Palakkad central plains

NS – No symptoms

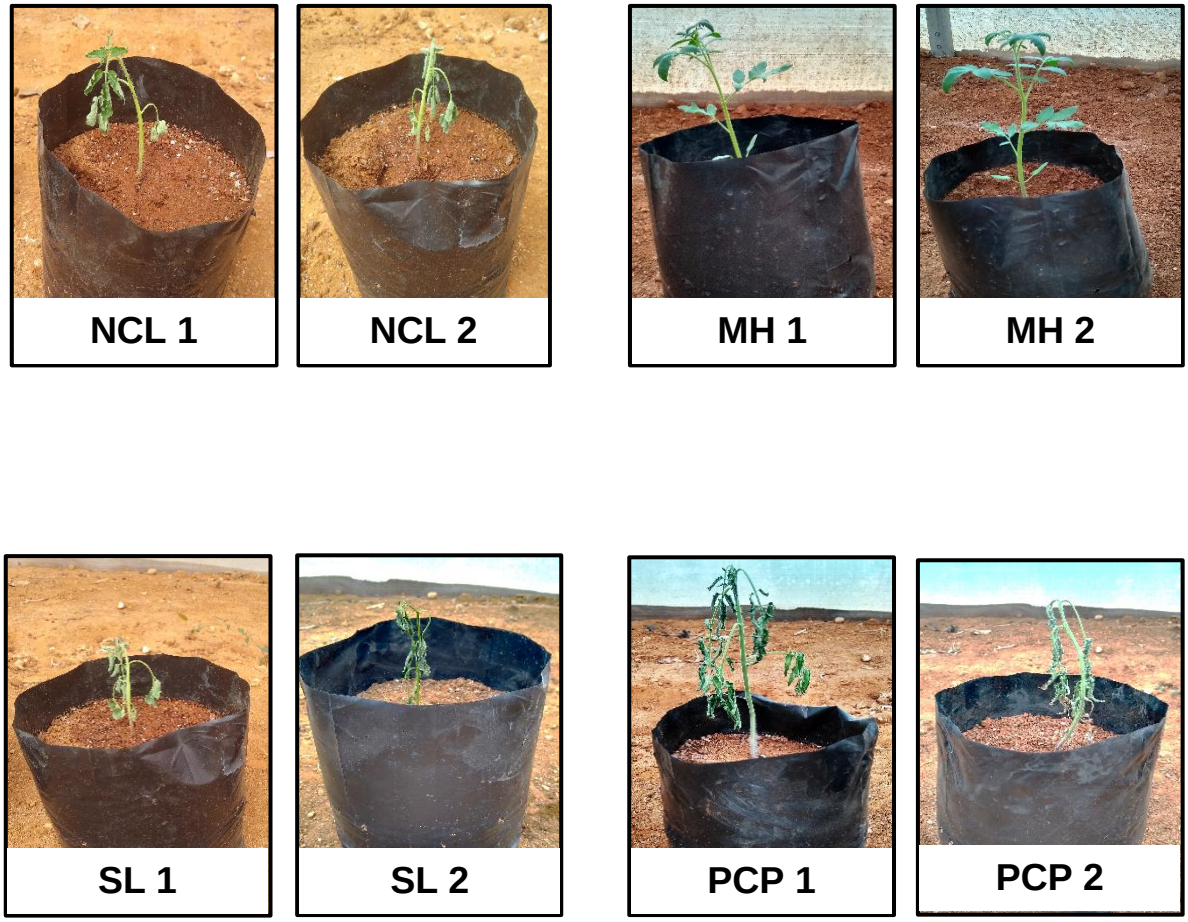


Plate 4.7 Reaction of tomato to isolates of *R. solanacearum*

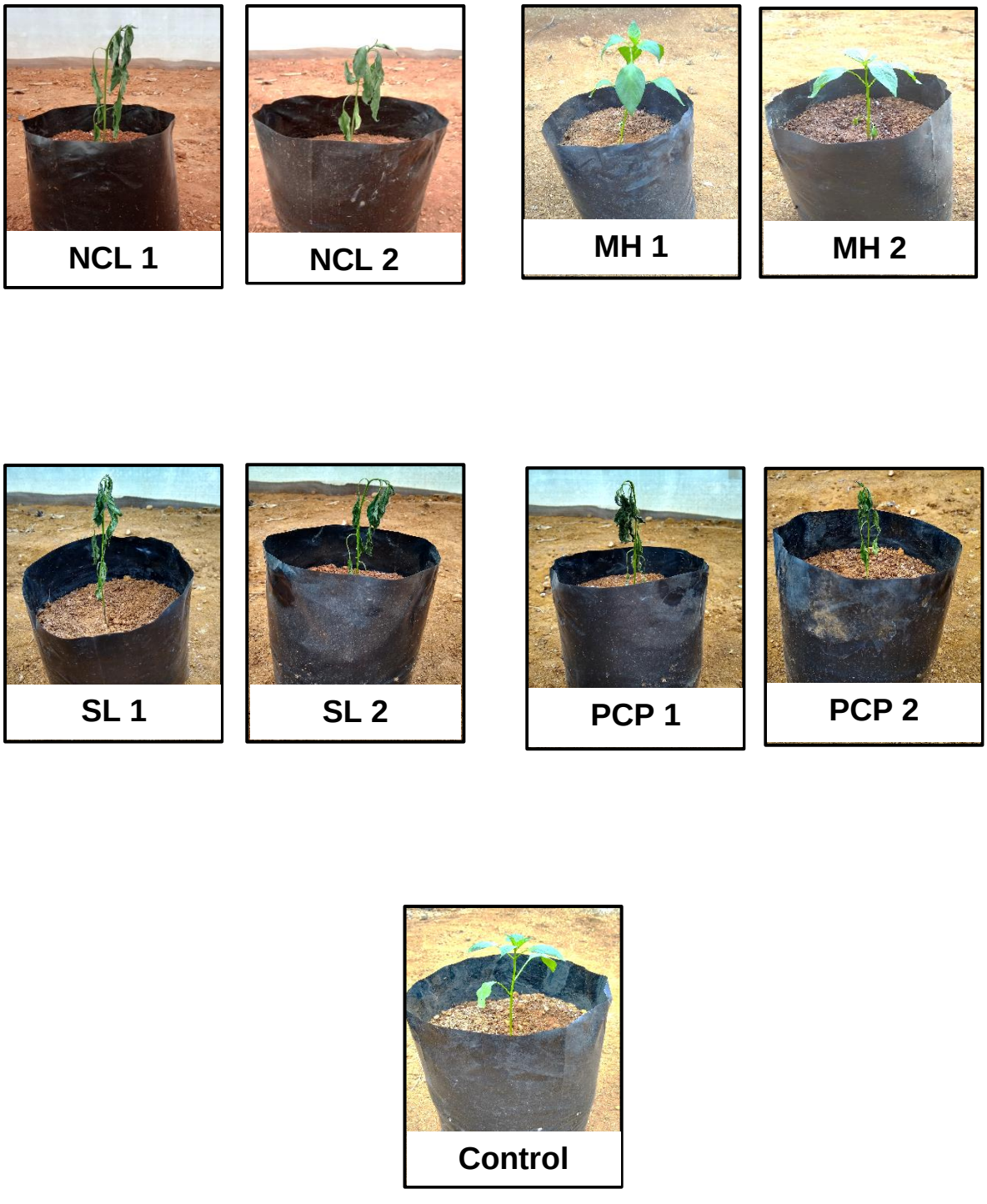


Plate 4.8 Reaction of chilli to isolates of *R. solanacearum*

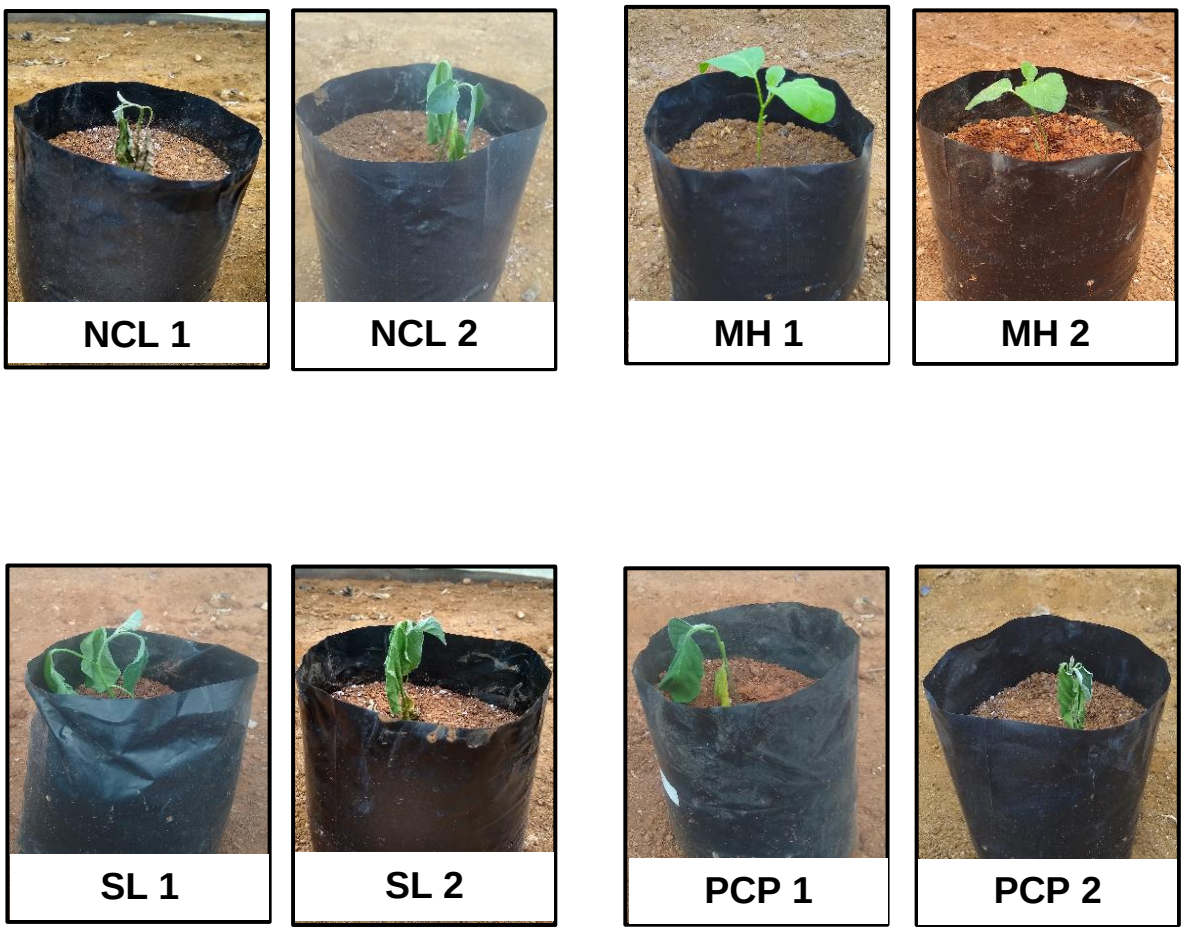


Plate 4.9 Reaction of brinjal to isolates of *R. solanacearum*

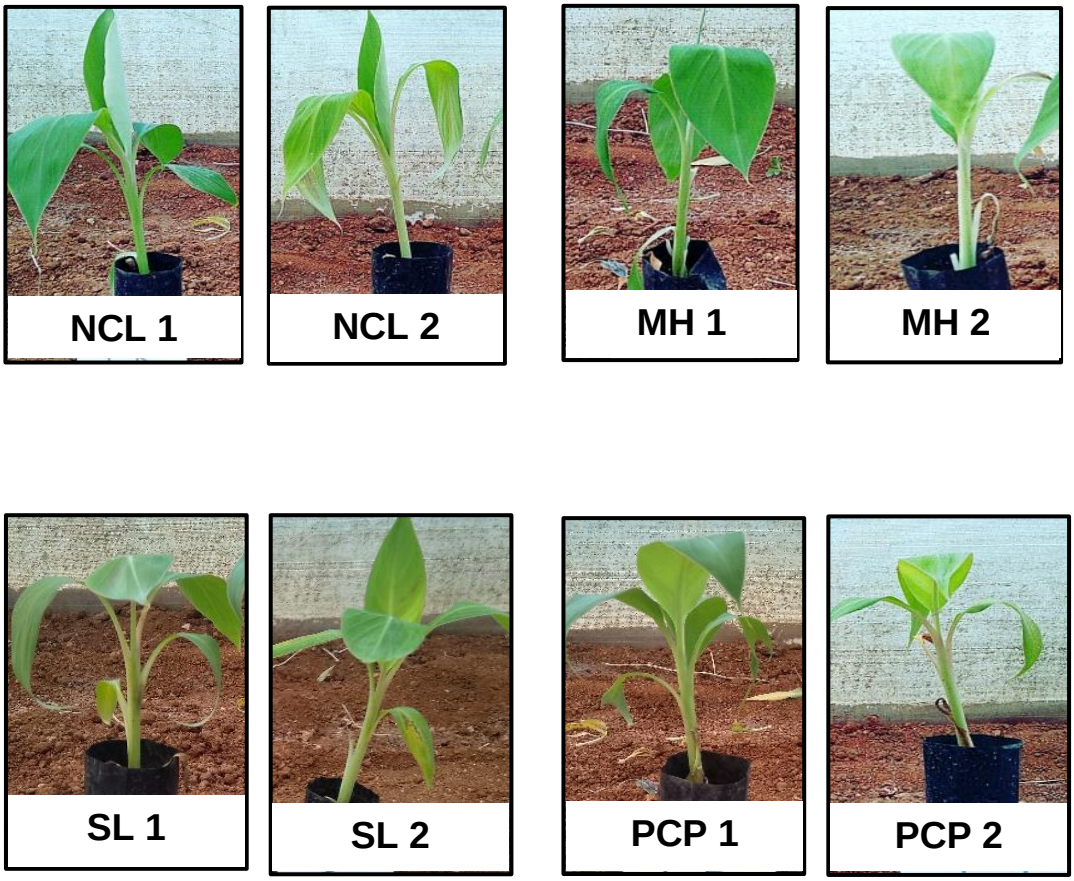
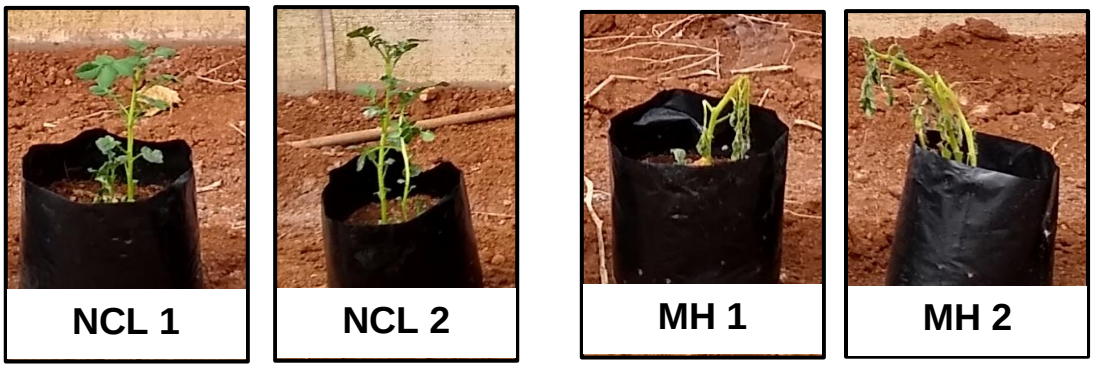


Plate 4.10 Reaction of banana to isolates of *R. solanacearum*



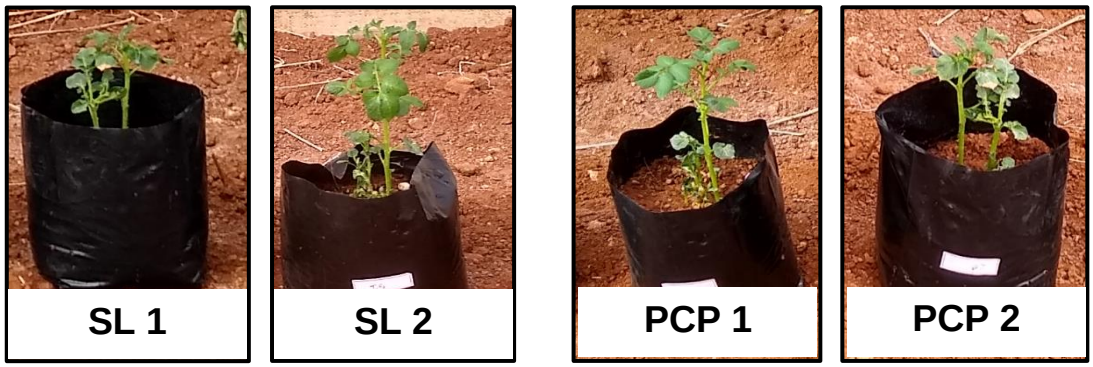


Plate 4.11 Reaction of potato to isolates of *R. solanacearum*

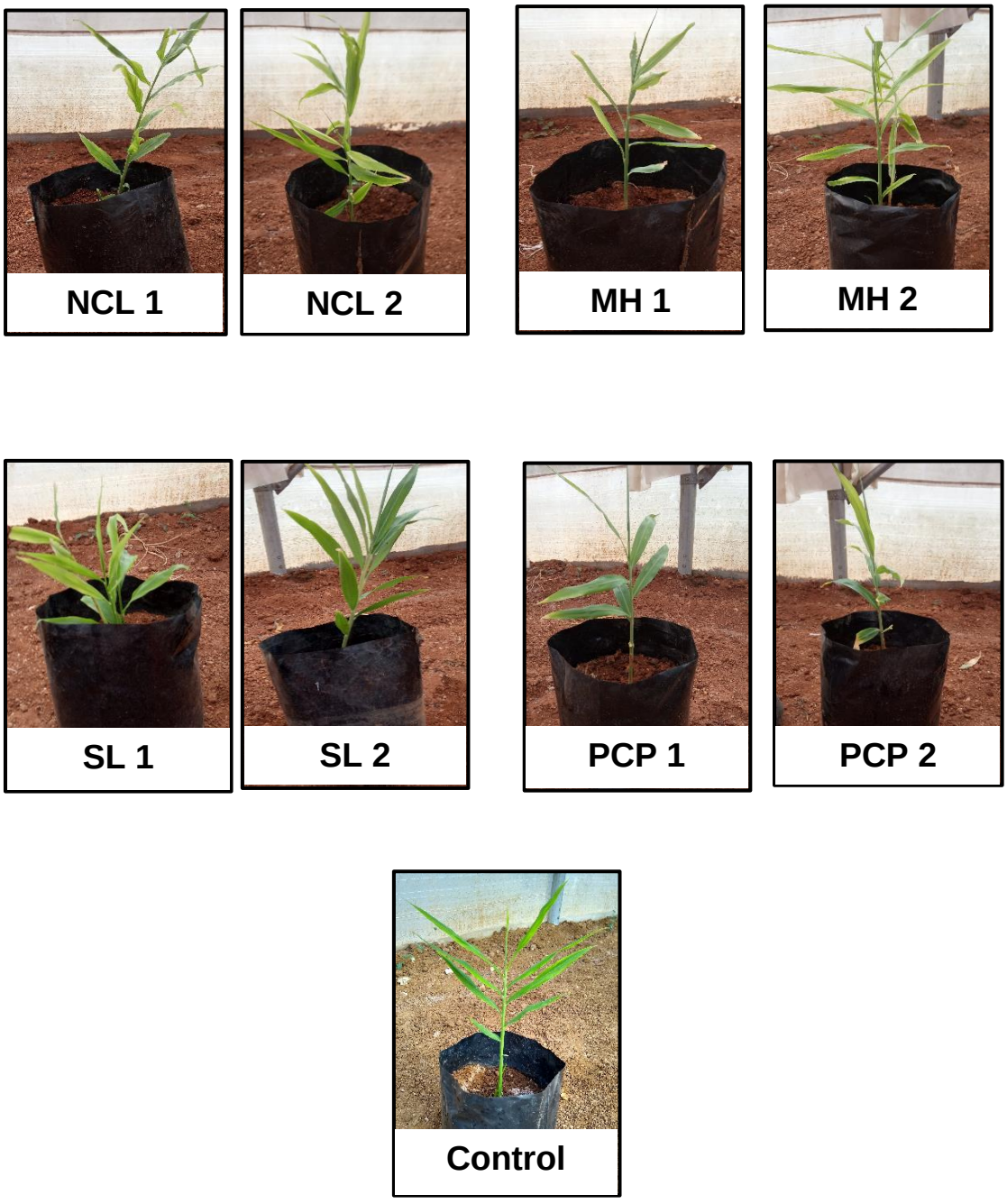


Plate 4.12 Reaction of ginger to isolates of *R. solanacearum*

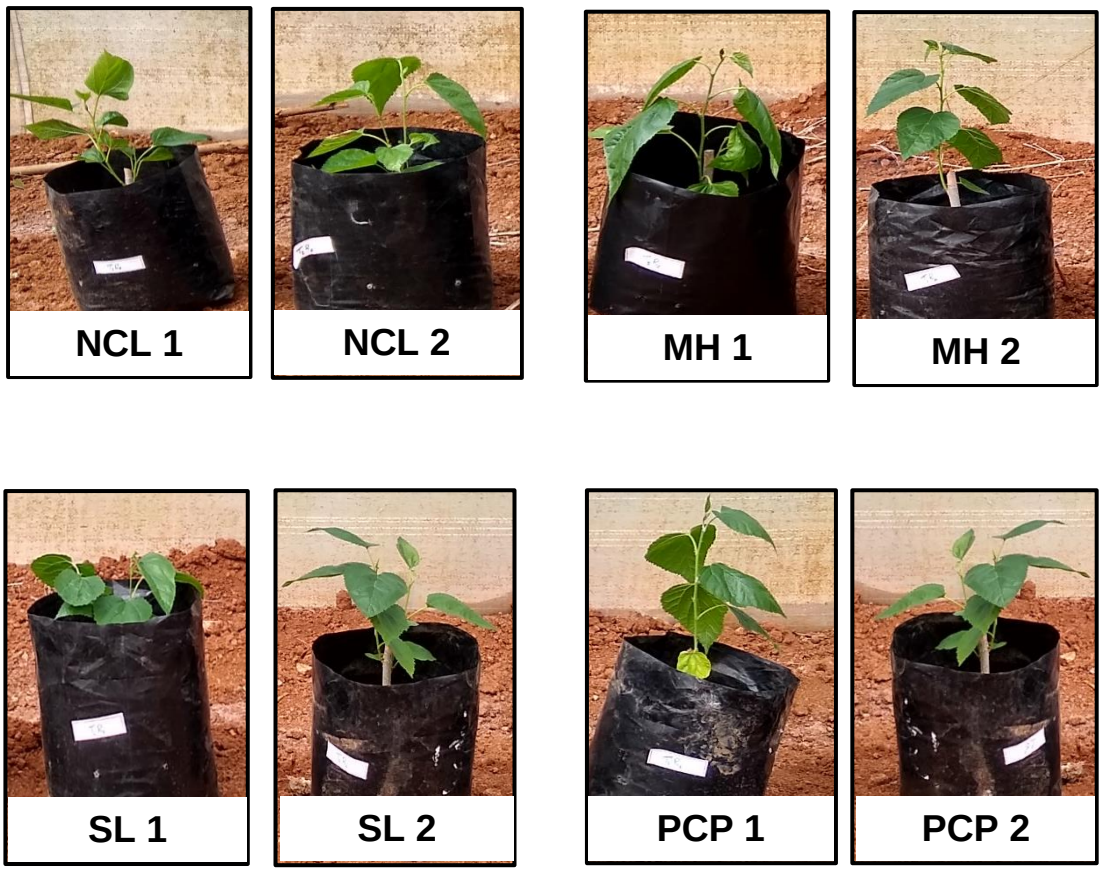


Plate 4.13 Reaction of mulberry to isolates of *R. solanacearum*

Table 4.13 Utilisation of disaccharides and sugar alcohols by isolates from NCL

Time period (Days)	Isolates	Cellobiose	Maltose	Lactose	Dulcitol	Mannitol	Sorbitol	Biovar
1	NCL 1	−	−	±	−	−	−	
	NCL 2	−	−	−	−	−	−	
2	NCL 1	±	−	+	−	±	±	
	NCL 2	±	±	±	−	±	±	
3	NCL 1	+	±	±	±	+	+	
	NCL 2	+	+	+	±	+	+	
4	NCL 1	+	+	+	+	+	+	III
	NCL 2	+	±	+	±	+	+	
7	NCL 1	+	+	+	+	+	+	
	NCL 2	+	+	+	+	+	+	III
14	NCL 1	+	+	+	+	+	+	
	NCL 2	+	+	+	+	+	+	

NCL – Northern central laterite

MH – Maryur hills

SL – Southern laterite

PCP – Palakkad central plains

+: Complete colour change

±: Partial colour change

− : No colour change

Table 4.14 Utilisation of disaccharides and sugar alcohols by isolates from MH

Time period (Days)	Isolates	Cellobiose	Maltose	Lactose	Dulcitol	Mannitol	Sorbitol	Biovar
1	MH 1	−	−	−	−	−	−	
	MH 2	−	−	−	−	−	−	
2	MH 1	−	−	−	−	−	−	
	MH 2	−	−	−	−	−	−	
3	MH 1	−	±	±	−	−	−	
	MH 2	−	−	−	−	−	−	
4	MH 1	±	±	±	−	−	−	
	MH 2	±	±	±	−	−	−	
7	MH 1	+	+	+	−	−	−	II
	MH 2	+	+	+	−	−	−	II
14	MH 1	+	+	+	−	−	−	
	MH 2	+	+	+	−	−	−	

NCL – Northern central laterite

MH – Maryur hills

SL – Southern laterite

PCP – Palakkad central plains

+: Complete colour change

±: Partial colour change

− : No colour change

Table 4.15 Utilisation of disaccharides and sugar alcohols by isolates from SL

Time period (Days)	Isolates	Cellobiose	Maltose	Lactose	Dulcitol	Mannitol	Sorbitol	Biovar
1	SL 1	±	−	−	−	±	−	
	SL 2	−	±	−	±	−	±	
2	SL 1	+	−	±	−	+	−	
	SL 2	±	+	±	+	+	+	
3	SL 1	+	±	+	±	+	+	
	SL 2	+	+	+	+	+	+	III
4	SL 1	+	+	+	+	+	+	III
	SL 2	+	+	+	+	+	+	
7	SL 1	+	+	+	+	+	+	
	SL 2	+	+	+	+	+	+	
14	SL 1	+	+	+	+	+	+	
	SL 2	+	+	+	+	+	+	

NCL – Northern central laterite

MH – Maryur hills

SL – Southern laterite

PCP – Palakkad central plains

+: Complete colour change

±: Partial colour change

− : No colour change

Table 4.16 Utilisation of disaccharides and sugar alcohols by isolates from PCP

Time period (Days)	Isolates	Cellobiose	Maltose	Lactose	Dulcitol	Mannitol	Sorbitol	Biovar
1	PCP 1	±	±	±	−	−	−	
	PCP2	±	−	±	−	−	±	
2	PCP 1	+	+	+	−	±	±	
	PCP 2	+	+	+	−	±	+	
3	PCP 1	+	+	+	−	+	+	
	PCP 2	+	+	+	−	+	+	
4	PCP 1	+	+	+	−	+	+	
	PCP 2	+	+	+	−	+	+	
7	PCP 1	+	+	+	−	+	+	
	PCP 2	+	+	+	−	+	+	
14	PCP 1	+	+	+	−	+	+	IIIA
	PCP 2	+	+	+	−	+	+	IIIA

NCL – Northern central laterite

MH – Marayur hills

SL – Southern laterite

PCP – Palakkad central plains

+: Complete color change

±: Partial color change

− : No color change

Table 4.17 Identification of biovars of *R. solanacearum* by carbohydrate utilisation test

Isolate	Cellobiose	Lactose	Maltose	Dulcitol	Mannitol	Sorbitol	Biovar
NCL 1	+	+	+	+	+	+	III
NCL 2	+	+	+	+	+	+	III
MH 1	+	+	+	-	-	-	II
MH 2	+	+	+	-	-	-	II
SL 1	+	+	+	+	+	+	III
SL 2	+	+	+	+	+	+	III
PCP 1	+	+	+	-	+	+	IIIA
PCP 2	+	+	+	-	+	+	IIIA

NCL – Northern central laterite

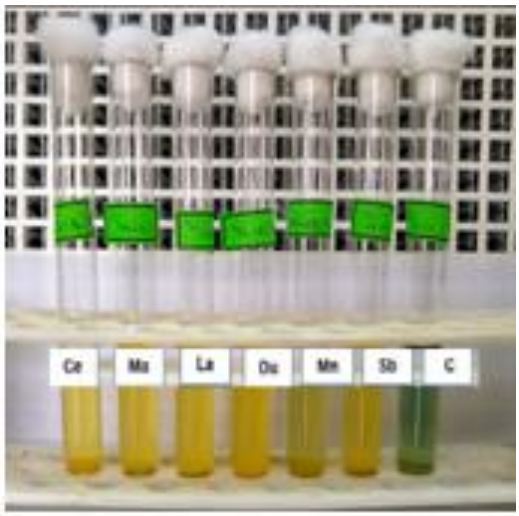
MH – Maryur hills

SL – Southern laterite

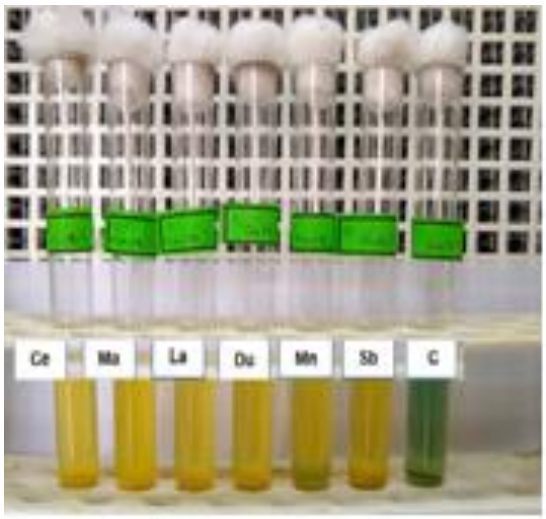
PCP – Palakkad central plains

\+ : Positive for sugar alcohol utilisation

– :Negative for sugar alcohol utilisation



NCL 2



NCL 1

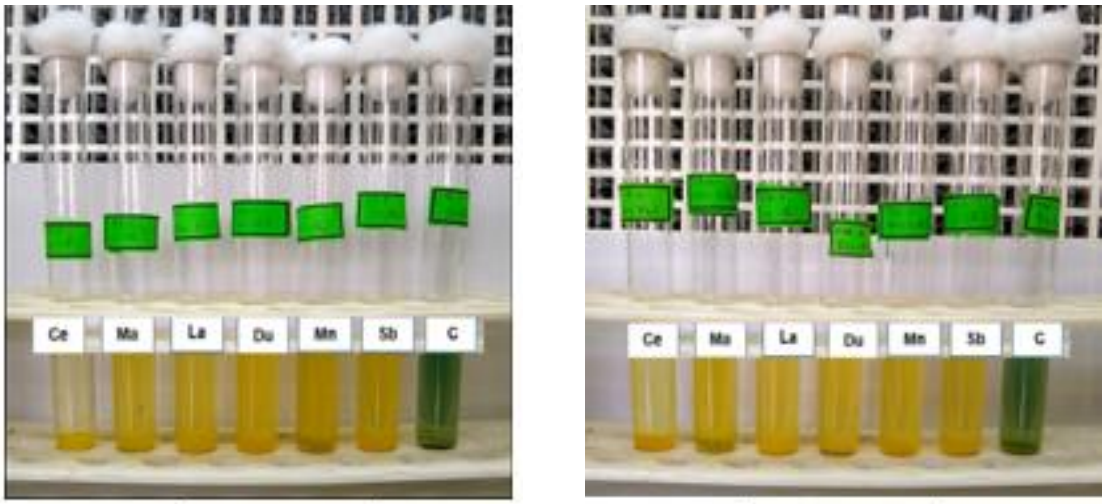
Biovar III

MH 1

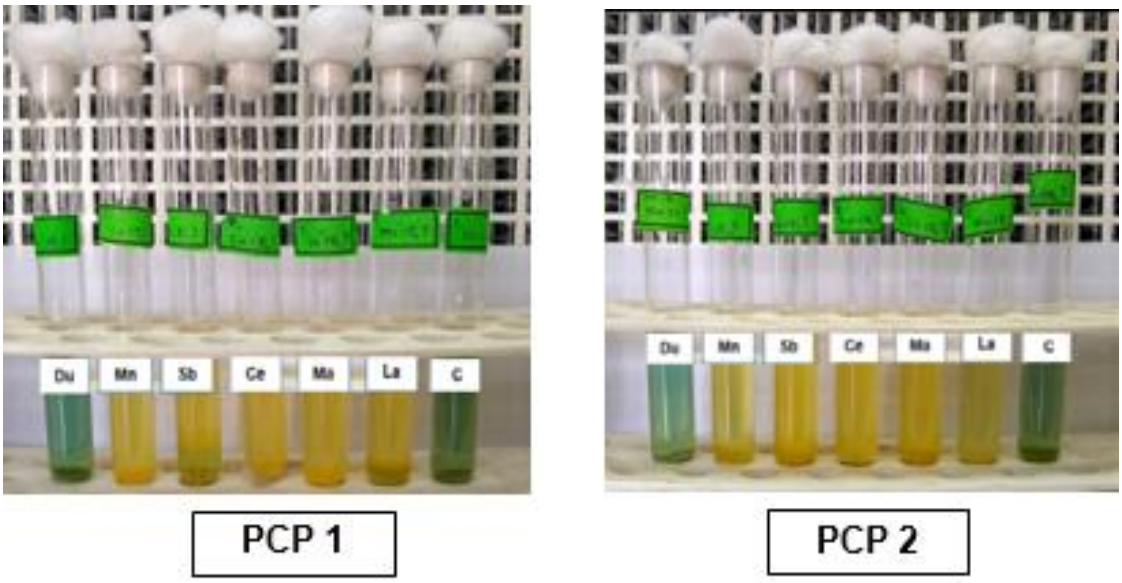
MH 2

Biovar II

Plate 4.15 Utilisation of disaccharides and hexose alcohols by isolates from NCL and MH



Biovar III



Biovar IIIA

Plate 4.15 Utilisation of disaccharides and hexose alcohols by isolates from SL and PCP

4.7 ANALYSIS OF PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF RHIZOSPHERE SOIL OF *R. solanacearum*

4.7.1 Estimation of physical properties of rhizosphere soil

The physical properties of rhizosphere soil of healthy and diseased plants were estimated using the procedure mentioned in section 3.7.1 (Table 4.18). The soil temperature ranged from 25.2°C to 37.8°C in the locations surveyed and the soil type was chiefly clay loam with exception of silty clay loam in PCP 2. The bulk density was within the optimum limit for plant growth, ranging from 1.04 to 1.29 g/cm^3 and water holding capacity varied from 38.66 per cent to 51.27 per cent.

The paired sample t-test was used to determine whether there is statistical evidence that the soil physical properties differed between rhizosphere soil of healthy and diseased plants (Table 4.20) and no significant difference was observed.

4.7.2 Estimation of chemical properties of rhizosphere soil

The chemical properties of the rhizosphere soil namely pH, organic carbon, available P, K, Ca and micronutrients Fe and Mn were analyzed as mentioned in section 3.7.2 (Table 4.18). Soil reaction was predominantly acidic with pH values in the range of 4.4 to 6.5. Organic carbon content varied from as low as 0.55 per cent in the rhizosphere soil of diseased plants of PCP 2 to as high as 2.39 per cent in the rhizosphere soil of healthy plants of MH 1. The available P content ranged from 67.31 kg ha^{-1} in the rhizosphere soil of diseased plants of PCP 2 to 600.78 kg ha^{-1} in the rhizosphere soil of diseased plants of NCL 1. The content of available K in these soils varied widely with the highest value being 2321.76 kg ha^{-1} in rhizosphere soil of healthy plants of PCP 1 and the lowest being 71.38 kg ha^{-1} for diseased plants of SL 2. The available Ca content also showed wide variation ranging from 2220.64 mg kg^{-1} in the rhizosphere soil of healthy plants of PCP 1 to 122.76 mg kg^{-1} in the rhizosphere soil of diseased plants of SL 2. The micronutrients in soil *viz.*, Fe content ranged from 156.2 mg kg^{-1} in the rhizosphere soil of healthy plants of SL 1 to 14.63 mg kg^{-1} in rhizosphere soil of diseased plants of SL 2 and Mn content

showed variation from 19.25 mg kg^{-1} in rhizosphere soil of diseased plants of SL 2 to 256.60 mg kg^{-1} rhizosphere soil of healthy plants of NCL 1.

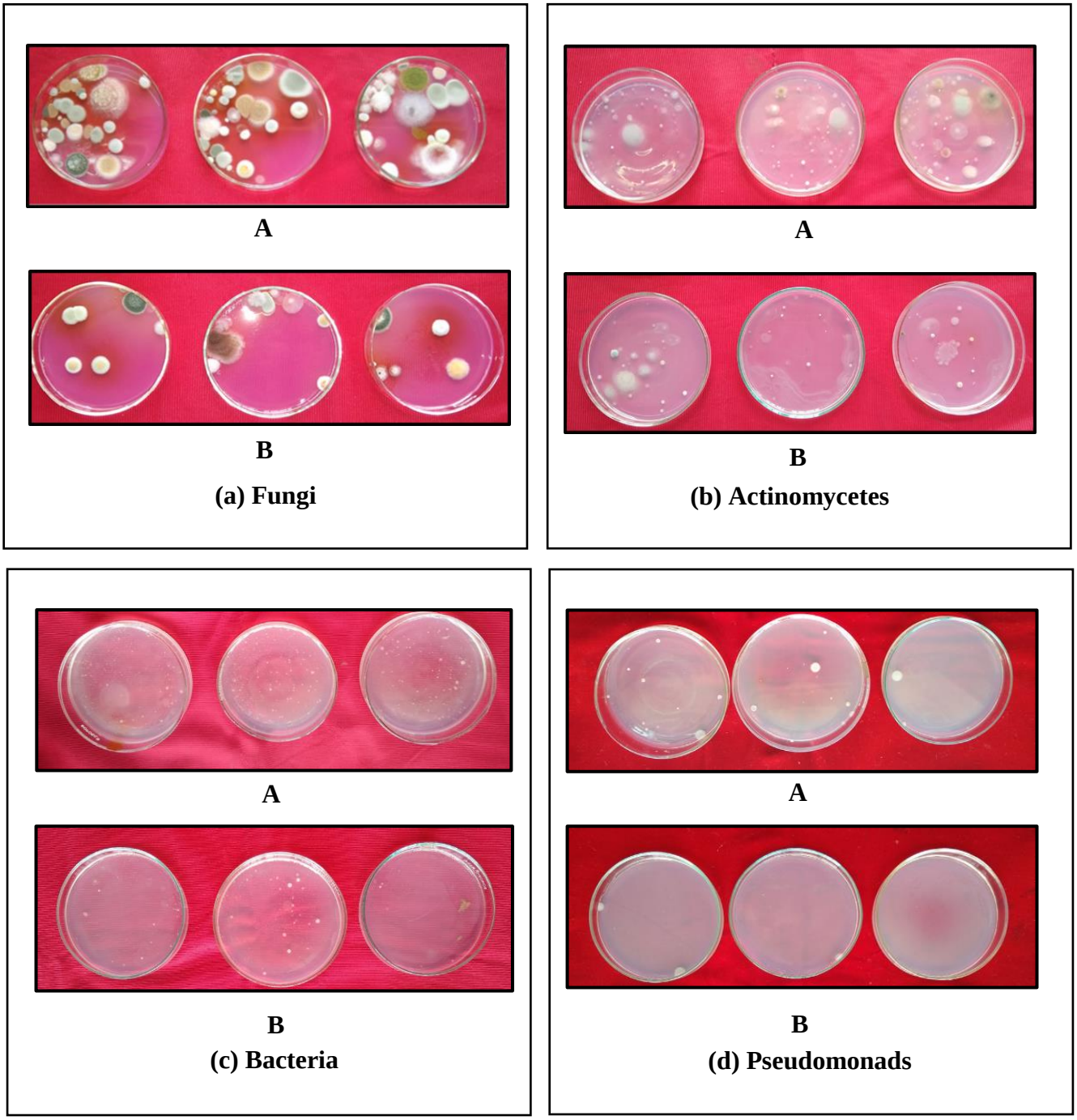
The statistical comparison using paired sample t-test revealed that pH, organic carbon, available K, Ca and Fe content are significantly higher in the rhizosphere soil of healthy plants compared to diseased (Table 4.20).

4.7.3 Enumeration of rhizosphere microflora

The quantitative estimation of soil microflora was carried out as mentioned in the section 3.7.3 (Table 4.19). In general, the population of rhizosphere microflora *viz.*, fungi, total bacteria, actinomycetes and fluorescent Pseudomonads was higher in the rhizosphere soil of healthy plants as compared to the diseased (Plate 4.16). This was further supported by results of paired sample t-test indicating significant difference in rhizosphere microflora of diseased and healthy plants (Table 4.20).

The per cent variation in population of total bacteria in the rhizosphere of healthy plants over diseased plants ranged from 19.14 per cent (NCL 1) to 72.93 per cent (SL 1) and it was found to be above 60 per cent in five of the eight locations. The population was significantly higher in healthy soils as the highest value for T- calculated (8.22) indicated a considerable difference (Table 4.20). This was followed by the population of fluorescent pseudomonads with a T- calculated value of 5.87 and the variation in population of these bacteria in the rhizosphere of healthy over diseased plants ranged from 85.34 per cent (PCP 1) to 38.46 per cent (MH 2).

However in the case of fungi, seven out of eight locations recorded higher population of microflora in the rhizosphere of healthy plants compared to diseased and the per cent variation ranged from 16.08 per cent (NCL 1) to 86.31 per cent (PCP 2). However, in the case of location PCP 1, the population of fungi was found to be lower in healthy compared to diseased. The population of actinomycetes was also found to be higher in the rhizosphere of healthy plants with the exception of PCP 1. The variation in population of actinomycetes ranged from 7.36 per cent (SL 2) to 82.52 per cent (NCL 1).



A – Rhizosphere microflora from healthy plants

B – Rhizosphere microflora from *R. solanacearum* infected plants

Plate 4.16 Enumeration of rhizosphere microflora of healthy and *R. solanacearum* infected plants

Table 4.18 Physico-chemical properties of rhizosphere soil of healthy and diseased plants

Location	Soil temperature (°C)	Soil texture	Bulk density (g/cm3)	Water holding capacity (%)	pH	Organic carbon (%)	Available P (kg/ha)	Available K (kg/ha)	Available Ca (mg/kg)	Fe (mg/kg)	Mn (mg/kg)
NCL 1 (Healthy)	36.7	Clay loam	1.16	49.99	4.8	1.27	417.57	400.96	794.75	39.88	256.60
NCL 1 (Diseased)	37.8	Clay loam	1.15	49.57	4.4	0.99	600.78	640.19	578.1	38.40	250.15
NCL 2 (Healthy)	32.4	Clay loam	1.29	38.66	5.5	1.40	482.70	1901.2	2350	99.97	158.85
NCL 2 (Diseased)	32.7	Clay loam	1.26	40.25	5.2	1.21	338.43	699.22	2133.75	90.80	104.25
MH1 (Healthy)	25.2	Clay loam	1.27	42.93	6.5	2.39	418.78	2170	3290	73.36	54.9
MH 1 (Diseased)	26.4	Clay loam	1.24	40.75	5.4	1.72	130.87	314.72	1402.45	37.98	31.4
MH2 (Healthy)	25.9	Clay loam	1.28	43.00	5.8	2.26	460.78	487.31	2581	67.23	79.95
MH 2 (Diseased)	28.8	Clay loam	1.26	45.70	5.6	1.27	102.26	416.64	1820	35.23	96.30
SL1 (Healthy)	31.1	Clay loam	1.09	48.18	5.2	1.48	73.33	935.2	479.36	156.2	28.25
SL 1 (Diseased)	30.2	Clay loam	1.09	49.33	4.9	0.84	83.58	364	143.36	149.0	28.90
SL2 (Healthy)	30.1	Clay loam	1.04	47.68	5.4	0.55	202.07	96.32	325	18.03	19.25
SL 2 (Diseased)	30.0	Clay loam	1.05	51.27	4.8	0.48	198.77	71.68	122.76	14.63	20.60
PCP1 (Healthy)	29.6	Clay loam	1.27	41.08	5.8	1.28	203.60	2321.76	2220.64	39.01	68.30
PCP 1 (Diseased)	30.3	Clay loam	1.28	42.76	5.6	0.45	221.05	1094.24	1952.22	25.87	46.05
PCP 2 (Healthy)	29.7	Silty clay loam	1.13	47.26	5.6	1.32	83.23	337.76	1218.42	146.2	25.75
PCP 2 (Diseased)	29.9	Silty clay loam	1.12	47.57	5.3	0.58	67.31	104.24	851.12	122.4	25.20

Table 4.19 Population of rhizosphere microflora of healthy and diseased plants

Location	Fungi (x10^3 cfu/g)			Bacteria (x10^6 cfu/g)			Actinomycetes (x10^5 cfu/g)			Fluorescent Pseudomonads (x10^7cfu/g)		
	HS*	DS**	Variation (%)	HS	DS	Variation (%)	HS	DS	Variation (%)	HS	DS	Variation (%)
NCL 1	28.6	24.00	16.08	35.00	28.30	19.14	13.33	2.33	82.52	11.00	4.33	60.63
NCL 2	33.00	27.33	17.18	10.33	3.33	67.76	36.00	27.6	23.33	16.33	2.66	83.71
MH 1	30.33	25.33	16.48	10.67	3.33	68.79	29.00	21.00	27.58	16.66	4.00	79.12
MH 2	25.33	10.00	60.52	22.00	15.00	31.81	21.33	11.33	46.88	13.33	8.00	38.46
SL 1	30.33	18.66	38.47	16.00	4.33	72.93	18.00	12.00	33.33	12.66	2.66	78.98
SL 2	34.00	17.00	50.00	22.00	16.00	27.27	22.67	21.00	7.36	15.67	3.66	76.64
PCP 1	45.66	52.00	-13.88	21.33	7.33	65.63	17.33	18.00	-3.86	11.33	1.66	85.34
PCP 2	87.66	12.00	86.31	11.33	4.33	61.78	32.33	12.00	62.88	1.66	1.00	39.70

*HS- Rhizosphere soil of healthy plants
**DS- Rhizosphere soil of diseased plants
Variation (%) – percentage variation in population in diseased soil over healthy soil

4.8 CORRELATION OF SOIL PROPERTIES WITH PDI AND PATHOGEN POPULATION

Correlation analysis of rhizosphere soil properties with PDI and pathogen population was performed and it was found that water holding capacity and bulk density are positively correlated whereas pH and Ca are negatively correlated with the disease (Table 4.21). The water holding capacity of rhizosphere soil was positively correlated with PDI and pathogen population with a correlation coefficient (r) of 0.799 and 0.834 respectively. Bulk density also showed significant positive correlation with PDI (r = 0.761) and pathogen population (r = 0.835). Among the chemical parameters studied, soil pH showed the highest level of negative correlation with PDI (r = -0.957). A significant correlation (r = -0.807) was also seen between pH and pathogen population in soil. Similarly, available Ca was also negatively correlated with PDI and pathogen population. The available Ca and per cent disease incidence in soil displayed a significant negative correlation (r = -0.876) and was negatively correlated with pathogen population (r = -0.899).

4.8.1 Step-up multiple regression analysis for prediction of bacterial wilt in solanaceous crops

The data on soil properties with high significant correlation to disease incidence and pathogen population were subjected to step-up multiple regression analysis so as to find out the most important parameters as well as to assess the per cent contribution by them. The independent variables taken were water holding capacity (X_1), bulk density (X_2), pH (X_3) and available Ca (X_4) and the dependent variables were PDI (Y_1) and pathogen population in soil (Y_2).

Multiple regression analysis for prediction of bacterial wilt in solanaceous crops and *Ralstonia solanacearum* population in soil

	Prediction equation for bacterial wilt incidence	R^2	Adjusted R^2
1.	$Y_1 = 372.45 – 62.43\ X_3$	0.915	0.901
2.	$Y_1 = 294.18 – 44.483\ X_3 – 0.13\ X_4$	0.977	0.968
	Prediction equation for built-up of pathogen population in soil	**R^2**	**Adjusted R^2**
1.	$Y_2 = 125.34 – 0.52\ X_4$	0.808	0.776
2.	$Y_2 = -310.31 – 0.36\ X_4 + 355.81\ X_2$	0.945	0.922

A very high coefficient of multiple determination (Adjusted R^2 = 0.968) indicating significant contribution of 96.8% in the prediction of bacterial wilt was recorded for pH and Ca. Similarly, bulk density and Ca content in rhizosphere soil contributed 92.2% in the build-up of population of pathogen in soil.

Table 4.20 Soil properties with significant effect on the disease

Sl No.	Soil properties	T calcuated
1.	Soil temperature	1.452
2.	Water holding capacity	2.286
3.	Core bulk density	1.140
4.	pH	3.991**
5.	Organic carbon	4.721**
6.	Available P	1.190
7.	Available K	2.372*
8.	Available Ca	2.606*
9.	Fe	3.404*
10.	Mn	1.438
11.	Fungus	2.642*
12.	Bacteria	8.217**
13.	Actinomycetes	2.693*
14.	Fluorescent Pseudomonads	5.869**

T- Table (0.05): 2.365, T- Table (0.01): 3.499

*- Significance at 5 per cent level

** - Significance at 1 per cent and 5 per cent level

Table 4.21 Correlation of soil properties with PDI and pathogen population

Sl No.	Soil properties	Correlation coefficient (r)	
		PDI	Pathogen population
1.	Soil temperature	0.443	0.375
2.	Water holding capacity	0.799*	0.834**
3.	Core bulk density	0.761*	0.835**
4.	pH	-0.957**	-0.807*
5.	Organic carbon	-0.260	-0.456
6.	Available P	0.430	0.193
7.	Available K	-0.395	0.338
8.	Available Ca	-0.876**	-0.899**
9.	Fe	0.048	0.094
10.	Mn	0.320	0.050
11.	Fungi	-0.253	-0.126
12.	Bacteria	0.580	0.416
13.	Actinomycetes	-0.284	-0.247
14.	Pseudomonads	-0.023	-0.103

*- Significance at 5 per cent level

** - Significance at 1 per cent and 5 per cent level

5. Discussion

5. DISCUSSION

Bacterial wilt caused by *R. solanacearum* is a disease of worldwide importance, affecting several economically important crops in tropical, subtropical and warm temperate regions. It has been ranked as the most devastating disease of solanaceous crops, due to the extreme complexity and diversity of the causal agent, *Ralstonia solanacearum.* Being a highly diverse pathogen, it has been described as a species complex (Fegan and Prior, 2005). The pathogen is systemic and soil borne causing yield loss up to 100 per cent depending on many factors including local climate, soil types, cropping practices, the choice of crop and the virulence of the pathogen.The survival and variability of *R. solanacearum* in soil is affected by physico-chemical and biological properties of soil and the combination of these determines the fate of the pathogen in soil. Considering these facts, present study aims to collect and characterise *R. solanacearum* isolates from four different agro ecological units of Kerala and to determine the influence of soil physical, chemical and biological properties on the pathogen and disease.

5.1 SURVEY AND COLLECTION OF BACTERIAL WILT SAMPLES

Purposive sampling survey was conducted in four agro ecological units (AEUs) of Kerala, and two locations in each AEU were included in the survey. The per cent disease incidence was recorded and it was found that, bacterial wilt incidence caused by *R. solanacearum* in different locations varied from 88 per cent in the location NCL 1 (Pudukkad) to 20 per cent in the location PCP 1 (Vithanassery). A comparatively lower wilt incidence was observed in both locations of Marayur hills, that is, 35.7% (MH 1- Perumalai) and 25.8% (MH 2- Kanthaloor) as the disease incidence was more prevalent in plains as compared to hills (Shekhawat *et al*., 1978). Though, bacterial wilt was prevalent in all the locations (Mathew and Peter, 2004), the disease intensity and population of the pathogen vary widely and this could be attributed to the variation in soil parameters.

In order to understand the extent of contribution of pathogen population in bacterial wilt incidence, it is essential to quantify the population of *Ralstonia solanacearum* in soil. The population of the pathogen in the rhizosphere soil of

diseased and healthy plants were quantified by serial dilution and plating on SMSA-E (Selective Medium South Africa - Elphinstone). SMSA-E is considered an effective medium for assessing the *R. solanacearum* population in soil (Pontes *et al*., 2017). Hence the use of this medium enabled an effective quantification of the pathogen in soil as it suppressed the growth of contaminating microorganisms, resulting in the lowest rate of repression of *R. solanacearum*. The population of the *R. solanacearum* present in the rhizosphere soil ranged from 20.66 x 10^4 cfu $g^{-1}$of rhizosphere soil of healthy plants of MH 2 (Kanthaloor) to 137.66 x 10^4 cfu g^{-1} of rhizosphere soil of diseased plants of SL 2 (Pravachambalam). A significant difference in pathogen population was observed between the rhizosphere soil of diseased and healthy plants of the same location. That is, the pathogen count was significantly lower in the rhizosphere of healthy plants as compared to diseased plants as observed earlier by Wang *et al*. (2017). Similar observations were noted by Liu *et al.* (2015) also, where *R. solanacearum* cells from rhizosphere soil of diseased tomato plants were higher as compared to healthy rhizosphere soil when plated on SMSA.

The population of the pathogen in the rhizosphere soil of diseased plants followed a trend similar to PDI in all the locations and they were positively correlated. The scatter diagram depicting a R^2 value of 0.848 indicated that 84.8 per cent of variation bacterial wilt incidence is explained by pathogen population present in the soil (Fig. 5.1). Hence, the more abundant the pathogen in rhizosphere soil, the more will be wilt incidence. The strong positive correlation exhibited by the pathogen population and PDI supports this conclusion. However, possible role played by variation in the virulence of the pathogen present in different locations cannot be ruled out as there are reports suggesting the influence of soil parameters like temperature and soil reaction on the virulence of *R. solanacearum* (Bocsanczy *et al*., 2012; Li *et al*., 2017).

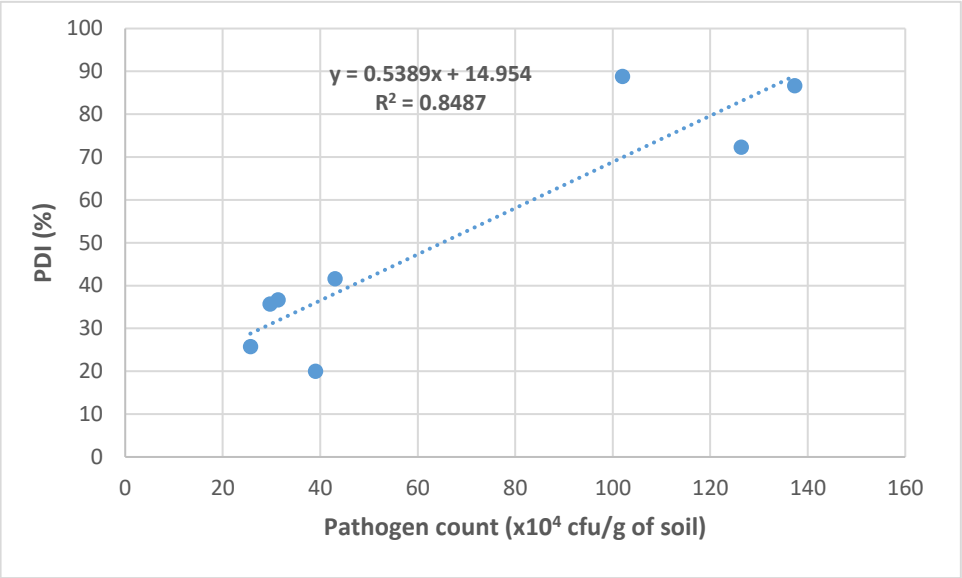


Fig. 5.1 Correlation between PDI and population of *R. solanacearum*

5.2 ISOLATION AND PATHOGENICITY TEST

The wilted plant samples collected from different locations were subjected to ooze test to confirm the presence of bacterium. Ooze test is commonly used to distinguish between fungal and bacterial wilt affected plants as the milky white ooze is known to contain bacterial cells (Chakravarty and Kalita, 2011). The transverse sections of infected potato tubers collected during the survey showed typical bangle blight symptom due to the necrosis of vascular tissue as observed by many workers (Butler, 1903; Hingorani *et al*., 1956; Mazzucchi, 1995). The cut sections of potato tubers when placed in sterile water exuded fine milky white ooze as observed by Ahmed *et al.* (2013). The pathogen was isolated from the bacterial ooze collected in sterile water from infected plant samples. This was streaked on TZC agar, which is the commonly used selective medium for the isolation and characterisation of *R. solanacearum*. Fluidal, creamy white colonies with reddish to pink center, characteristic of *R. solanacearum* appeared after 48 h. of incubation at room temperature (Chaudhry and Rashid, 2011).

The pathogenicity of the bacterium isolated from the infected plants was established by artificial inoculation of freshly collected bacterial ooze to the respective host plants. The inoculated plants expressed characteristic bacterial wilting symptoms and the presence of the bacterium in them was confirmed by ooze test followed by re-isolation on TZC agar. The colonies formed on TZC agar were identical to those obtained from wilted plants collected in the survey, thus confirming the pathogenicity of the bacterium.

After isolation and purification, the bacterial isolates were maintained in sterile distilled water at room temperature in screw capped glass vials to maintain the viability. Bacterial stock cultures maintained in sterile distilled water at room temperatures was found to retain the viability up to a period of one year (He *et al*., 1983; Prior and Steva, 1990; Mathew *et al*., 2000). In the present study, in order to avoid the development of non-mucoid mutant colonies and loss of virulence, the preserved cultures were sub-cultured once in two months as suggested by James (2001).

5.3 CHARACTERISATION OF THE ISOLATES

5.3.1 Cultural characterisation

The cultural characters of the eight isolates of *R. solanacearum* obtained from the different locations of the agro ecological units were studied on TZC agar. All the isolates produced circular colonies with entire margin. The colonies were slightly raised except for PCP 1 and PCP 2 where the colonies were flat. The size of the colony observed were in close conformity with the observations of Pawaskar *et al*. (2014) and varied from pin point (1.5 mm) to moderate (5.5 mm). The fluidity of the colony varied among the isolates. The isolates of Palakkad central plains produced dark red colored colonies with high fluidity and dirty white margin (Lemessa and Zeller, 2007). Whereas, those of Northern central laterite and Southern laterite were creamy white with a pink center and appeared moderately fluid. Isolates of *R. solanacearum* with similar colony characters were collected from elsewhere by many researchers (Khan, 1974; He *et al.,* 1983; Prior and Steva, 1990; Singh *et al*., 2010 and Sahu *et al*. 2013). However, the isolates of the bacterium collected from infected potato plants from Marayur hills, showed a slight variation in cultural characters. They were pin point (1.5mm) to small size (1.5-3 mm) and were moderately fluidal with a light pink center as observed by Hussain (1995) and Dhital *et al*. (2001) when the pathogen was isolated from wilt affected potato plants. Even though the eight isolates collected in the present study were distinct with slight variations in colony characters, the deviations are within the limit of the typical characteristics of *R. solanacearum*. Moreover, the colour of the colonies varied from light pink to red with moderate to high fluidity indicating that all the isolates are virulent (Kumar *et al*., 2017).

5.3.2 Morphological characterisation

The morphological characterisation was carried out using Gram's staining and scanning electron microscopy (SEM). All the isolates showed negative reaction to Gram's staining (Rath and Addy, 1977; Khetmalas, 1984; Venkatesh, 1988; Chaudhry and Rashid, 2011; Pawaskar *et al*., 2014). Morphology of bacterial cells was studied using scanning electron microscope as per the protocol of Li *et al.* (2016). The protocol prerequisites an initial fixing of bacterial cells in glutaraldehyde (2.5%) overnight to preserve the cell integrity and structure. A slight modification was made in this

procedure where the duration of fixation in glutaraldehyde was reduced to two hours. Further, according to Li *et al*. (2016), images with high resolution were obtained at a working distance of 7.8 mm with a magnification of 20 kX. However, in this case, high quality images with the best resolution were obtained at 17.85 mm working distance with a magnification of 16.2 kX and at 8.98 mm with a magnification of 44.9 kX. Typical rod shaped cells of *R. solanacearum* of size 0.3-0.5µm x 1.2-1.7 µm were observed. The morphology of *Ralstonia solanacearum* cells has been studied by several workers employing scanning electron microscopy recording similar observations (Shoba, 2002; Narasimha and Srinivas, 2012; Pawaskar *et al*., 2014; Kumar, 2017; Teli *et al*., 2018).

5.3.3 Molecular characterisation

The 16S rDNA gene sequence due to its highly conserved nature marks evolutionary distance and relationship among bacteria (Kimura, 1980). Hence, the molecular characterisation of the isolates from different agro ecological units of Kerala was performed by PCR amplification of 16S rDNA gene of the bacterial DNA. *In-silico* analysis of the sequence data confirmed that all the isolates belong to *R. solanacearum* showing 99 to 100% homology with the reported sequences of the bacterium available in NCBI database. A sequence similarity of more than or equal to 99 per cent homology confirms the identity of bacteria at species level (Seal *et al.*, 1992).

The genetic diversity of *R. solanacearum* is widely reported from varying agro-climatic and geographical regions of the country. Many techniques *viz*. rep-PCR, PCR-RFLP, AFLP, PFGE and sequencing of virulence and other genes are being used to study the genetic diversity of *R. solanacearum* (Kumar *et al*. 2004; Smith *et al*. 1995; Jeong *et al*. 2007 and Stevens and Elsas, 2010). Phylogenetic analysis of 16S rDNA sequences can be used to configure variability among the isolates of *R. solanacearum* collected from different locations (Jeong *et al*., 2007). In the present study, the 16S rDNA sequences of the isolates were used to construct the phylogenetic tree and it was found that the isolates collected from different locations clustered on different branches of the phylogenetic tree suggesting considerable variation among them in accordance with the location. The phylogenetic tree branched into two major clusters

and all the isolates except those from Marayur hills were grouped into one cluster. Whereas, the isolates of Marayur hills clustered separately, this further confirms their discreet nature as compared to the others. The locations from where the isolates were collected belong to different AEUs of the state which are distinct with respect to soil type. *R. solanacearum*, being a soil-borne pathogen is easily influenced by the differences in soil environment and has developed specialised systems of complex regulatory cascades and networks which sense these differences and trigger changes in the physiology and gene expression (Agrios, 2005). Hence the variability among the isolates as well as similarity between two isolates from same AEU suggest that soil parameters play a significant role in the diversity expressed by the pathogen. Even though, Jaunet and Wang in 1999 could not relate genotypic variation of *R. solanacearum* to geographical origin, many recent studies strongly suggest otherwise. Among the 57 isolates of *R. solanacearum* collected from different locations of India by Chandrasekara *et al*. (2012), the isolates from Kerala were distinct from other isolates which suggests its variability in accordance with the location. Moreover, Chandrasekhar and Umesha (2015) analysed hrp B gene sequence of twenty isolates of *R. solanacearum* collected from Kerala and Karnataka and observed a clear and high degree of phylogenetic divergence among the isolates according to geographic region. Similarly, grouping of *R. solanacearum* isolates based on geographical location was done by Ivey *et al*. (2007) and Ramesh *et al*. (2014). Although the isolates from the same AEUs clustered together, the isolates from North Central laterite were seen in two different sub-clusters within the same cluster in the phylogenetic tree. The isolate NCL 1 showed more similarity to SL 2 while NCL 2 was more related to PCP 1. This variation maybe due to difference in soil parameters between the two locations within the same agro ecological unit. Furthermore, unlike isolates from other locations, NCL 1 and NCL 2 also differed with respect to host plant from which it was isolated.

5.4 CLASSIFICATION OF *Ralstonia solanacearum* ISOLATES INTO RACES AND BIOVARS

5.4.1 Detection of races

As early as in 1962, *R. solanacearum* has been divided into races based on host range. According to Buddenhagen *et al.,* race 1 infects many solanaceous plants such as brinjal, tomato, tobacco, pepper and other plants including some weeds while race 2 caused wilt of triploid banana (*Musa* spp.) and *Heliconia* spp. Race 3 affects potato and tomato but it is weakly virulent on other solanaceous crops. Further, Aragaki and Quinon (1965) reported the presence of race 4 infecting ginger and He *et al*. (1983) reported race 5 infecting mulberry. All the five races of *R. solanacearum* differ in host range, geographical distribution and ability to survive under different environmental conditions (Persley, 1986). Hence, infectivity of *R. solanacearum* isolates on host differentials is being employed to determine the race of the pathogen (Denny and Hayward, 2001; Chandrasekara *et al*., 2012, Kumar *et al*., 2017).

In the present study all the eight isolates collected from different locations were subjected to race differentiation by artificial inoculation into differential host plants. The results showed that the isolates obtained from Northern central laterite, Southern laterite and Palakkad central plains were able to cause symptoms on tomato, brinjal and chilli but not on other differential hosts, thus categorizing them as race 1. Race 1 of *R. solanacearum* has been earlier reported from Kerala by James *et al*. (2006). Moreover, all the 57 isolates of *R. solanacearum* obtained from different agro climatic zones of Karnataka and other parts of India were also classified as race 1. However, the isolates collected from Marayur hills produced symptoms on potato. But it failed to infect other solanaceous crops. So these cannot be classified into race 1. Further, it was not pathogenic on any other differential hosts other than potato. Hence it was designated as race 3. *R. solanacearum* race 3 infecting only potato has been earlier observed by Ahmed *et al.* (2013) from Bangladesh. Earlier in the present study it was observed that the isolates of Marayur hills exhibited distinct variation in cultural characteristics and formed a separate cluster in the phylogenetic tree (Fig. 4.1). The result of pathogenicity tests on host differentials further confirmed the discreet nature of these two isolates.

5.4.2 Identification of biovars

The different isolates of *Ralstonia solanacearum* are classified into biovars based on their ability to utilize disaccharides and sugar alcohols (Hayward, 1964). It was observed that, the eight isolates collected during the study differed in their ability to utilize alcoholic sugars and disaccharides. Accordingly, they were classified into biovars II, III and III A. The variation exhibited by the different isolates in the utilisation of sugars might be an indication of their host adaptability which enables them to utilize different sugars. Afroz *et al*. (2014) ascertained that inducible sugar utilization pathway in bacteria exhibits diverse single cell responses and this could be one of the probable reasons for the differential utilization of carbohydrates by the different biovars.

The isolates of brinjal and tomato of Northern central laterite and Southern laterite utilized all the carbohydrates, thus they were grouped into biovar III. Whereas the isolates obtained from Palakkad central plains utilized all the carbohydrates except dulcitol, hence grouped as biovar III A, a subtype of biovar III. Presence of biovar III and III A has been earlier reported from Kerala by James *et al*. (2001) and from Himachal Pradesh by Kumar *et al*. (1993). The isolates obtained from Marayur hills utilised disaccharides but failed to oxidise sugar alcohols and were classified as biovar II. The isolates of race 3 may correspond to biovar II (Buddenhagen, 1985) and in the present study also, the isolates collected from Marayur hills belonged to race 3 and biovar II. *R.* solanacearum race 3, biovar II infecting potato tubers has been reported by many workers (Dhital *et al*., 2001; Williamson *et al*., 2002; Messiha *et al*., 2007).

Hence based on the observations on race and biovar assessment as well as cultural and molecular characterisation of different isolates, it can be inferred that there is diversity existing within the population of *R. solanacearum* among the locations. This variability is contributed by the soil environment which harbour the pathogen. Being a successful soil inhabitant, *R. solanacearum* has developed specialised systems of complex regulatory cascades and networks which sense the difference in soil environment and trigger changes in the physiology and gene expression. It is proved that this mechanism fine-tunes the virulence and pathogenicity gene expression of

R. solanacearum (Agrios, 2005; Ivey *et al*., 2007; Ramesh *et al*. 2014; Chandrasekhar and Umesha, 2015).

5.5 INFLUENCE OF SOIL PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES ON BACTERIAL WILT INCIDENCE AND PATHOGEN POPULATION

One of the major objective of the study was to determine the relationship of soil physico-chemical and biological properties with the incidence of bacterial wilt. The results of the earlier experiments in the present investigation indicate that the soil properties play a major role in determining the diversity of the pathogen. Soil is an important repository for many plant pathogens and the survival of these pathogens in the absence of their specific host plants depends on a complex interplay between physical, chemical and biological factors in the soil. In the case of *Ralstonia solanacearum*, the soil climate is an important factor for survival and subsequent infection of host. Being a soil-borne plant disease, the occurrence and severity of bacterial wilt are tightly correlated with the physical and chemical properties of soil (Li *et al.*, 2017).

5.5.1 Soil physical properties

5.5.1.1 Soil Temperature

Soil temperature is an important factor influencing the multiplication of soil borne bacteria and a mean annual soil temperature in the range of 25 °C to 37°C was conducive to bacterial wilt (Smith, 1896). In accordance with this, in the present study the soil temperature at the locations surveyed ranged from 25.2 °C to 37.8 °C which indicates that there is distinct temperature requirement for disease development and reproduction of the pathogen (Swanepoel, 1990). Also, the pathogen prefers the humid tropical climate and high soil temperature (28-36 °C) prevailing in Kerala (Mathew and Peter, 2004). Of the locations surveyed, the potato fields of Marayur hills enjoy a cool climate during most of the period of the year. However, soil temperature in the range of 25.2°C to 28.8°C was recorded during the period of survey. An increase in soil temperature from 21°C to 35°C favoured the development of bacterial wilt in

potato (Hingorani *et al.,* 1956) accordingly, a per cent disease incidence up to 35.7 was recorded at Marayur hills.

5.5.1.2 Soil texture

The influence of soil type on disease severity has been repeatedly established (Moffett *et al*.,1983; Elsas *et al.,* 2000) and the disease is most severe on heavy clay-loam soil (Kelman, 1953). In the present study, irrespective of changes in crop and climatic conditions prevailing, the occurrence of bacterial wilt in all the locations maybe due to the presence of clay loam soil. This is supported by the findings of Keshwal *et al*., (2000) that elevated disease levels were expressed in clay soils as it harbored maximum population of bacterial wilt pathogen. Also, soil type and moisture levels individually and in combination had a significant effect on the severity of bacterial wilt as the disease severity was highest in clay soil (Abdullah *et al.*, 1983).

5.5.1.3 Water holding capacity

Soil moisture is known to have a profound influence on the rate of multiplication as well as movement of soil borne bacteria. The wilt incidence and population of pathogen in soil was found be to increasing with increase in water holding capacity in the rhizosphere soil of diseased plants in all locations. A significant positive correlation was obtained between water holding capacity and bacterial wilt incidence as well as pathogen population in soil. (Table 4.22). A per cent variation of 63.8 and 69.6 respectively in PDI and the population is due to difference in water holding capacity (Fig. 5.2 and 5.3). The increased incidence of the disease in soils with high water holding capacity can be attributed to the fact that high moisture in the soil will enhance the transpirational flow, thus facilitating the upward spread of pathogen in the infected plant (Gupta *et al.,* 2018). These were in corroboration with the findings of Keshwal *et al.* (2000) who reported that water holding capacity shows a highly significant positive correlation with pathogen population and disease index. Several other reports also confirm the role of water holding capacity on bacterial wilt development (Hingorani *et al.*, 1956; Sabet and Baraket, 1971).

5.5.1.4 Bulk density

The values of bulk density of the rhizosphere soil ranged between 1.04 g/cm^3 to 1.29 g/cm^3 and within this range an increase in bulk density resulted in increased wilt incidence and pathogen population and there was positive correlation among them (Table 4.22). The per cent contribution of this parameter on PDI and the population is 57.9 and 69.6 respectively (Fig. 5.4 and 5.5). This can be attributed to various factors such as soils with low bulk density supports the growth and multiplication of beneficial microflora (Li *et al.,* 2017) which in turn suppresses the pathogen and hence the disease. Moreover, availability of various nutrients especially Ca in soil is negatively correlated to bulk density which predisposes the plants to bacterial wilt Chaudari *et al*., 2013).

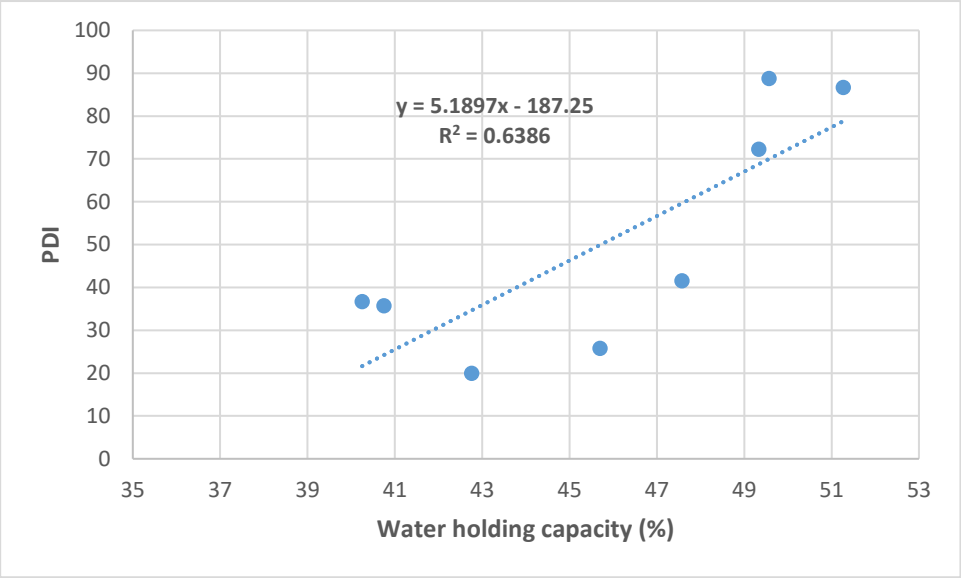


Fig. 5.2 Correlation between water holding capacity and PDI

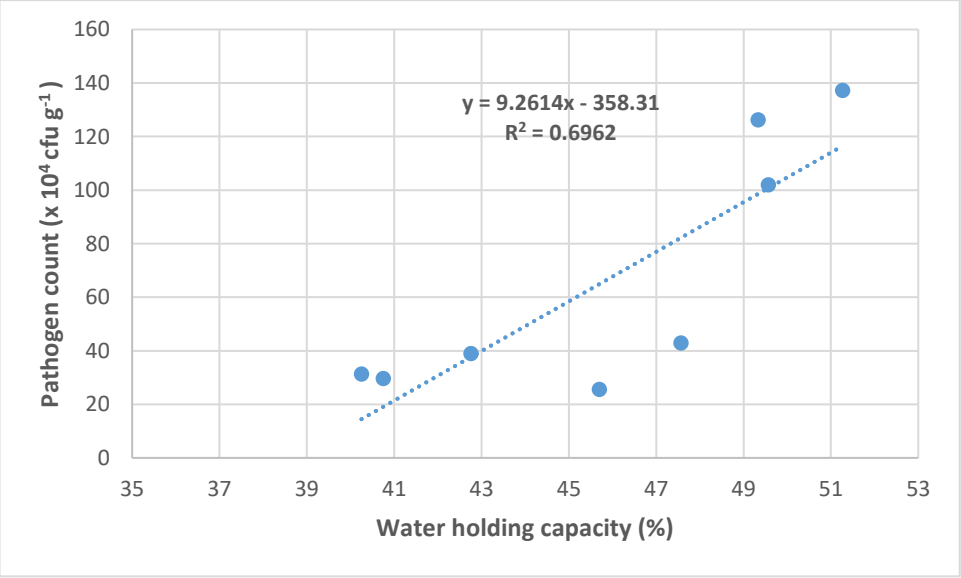


Fig. 5.3 Correlation between water holding capacity and population of *R. solanacearum*

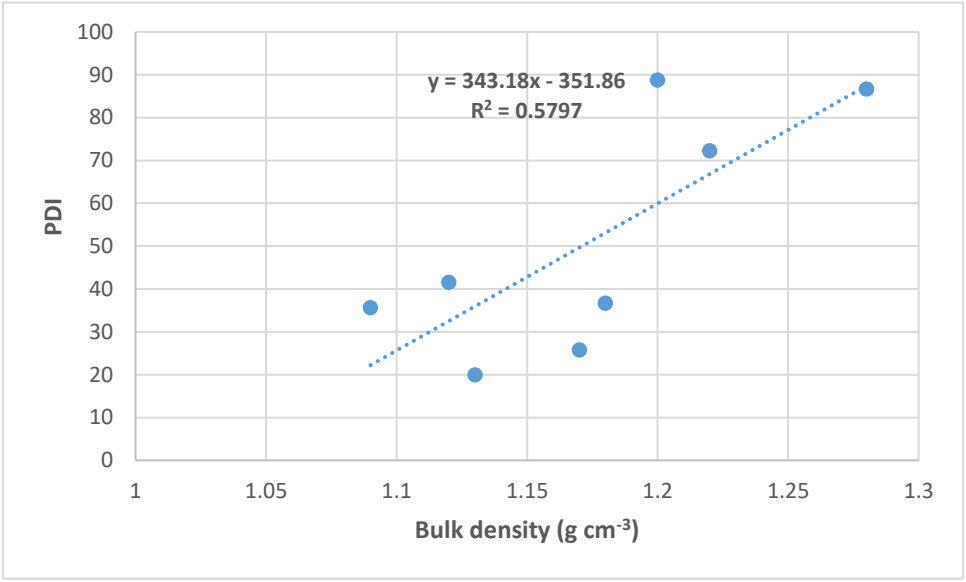


Fig.5.4 Correlation between bulk density and PDI

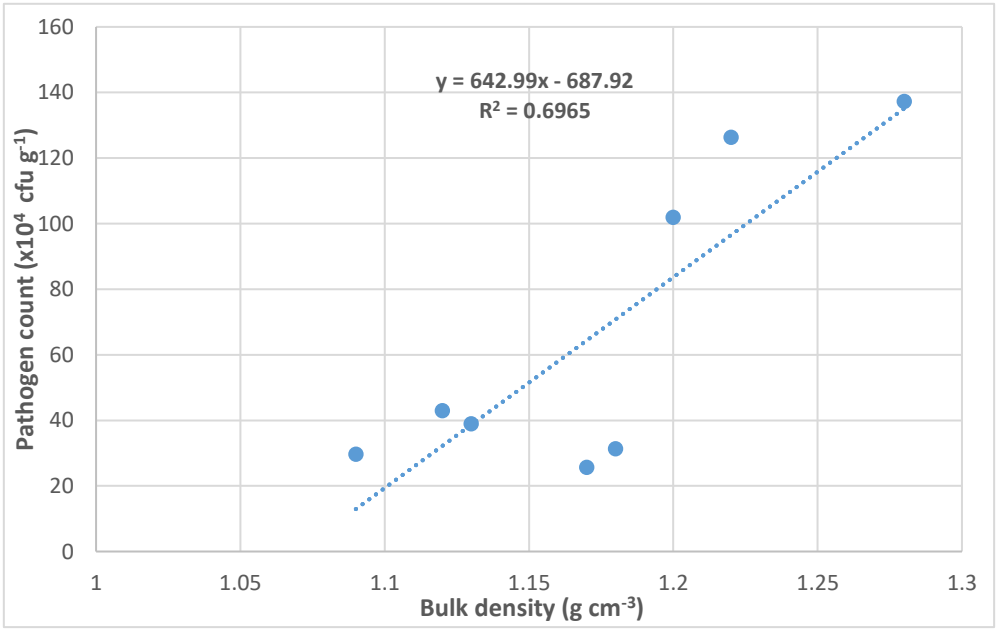


Fig. 5.5 Correlation between bulk density and population of *R. solanacearum*

5.5.2 Soil chemical properties

Nutrients are important for the growth and development of both plants and microbes, and nutrient deficiencies in soils weaken plants, making them more vulnerable to diseases (Agrios 2005). Several studies have shown that soil pH value, organic carbon content, total N, total P and total K content are negatively correlated with the population of soil-borne pathogens and the severity of diseases (Wang *et al*., 2017).

5.5.2.1 Soil pH

The ambient pH levels determine the capability of the pathogen to colonize, invade, and kill the host successfully (Alkan *et al.*, 2013). In the present study, soil pH was found to be lower in rhizosphere soil of diseased plants as compared to healthy plants. Soil reaction in the rhizosphere is usually more acidic in the case of diseased plants (Zheng *et al.* 2014, Wang *et al.* 2017). This is attributed to the fact that pathogen loses virulence rapidly at high pH and hence soils with high pH remains healthy (Shekhawat and Perombelon, 1991). The bacterial wilt incidence and pathogen population tend to increase with decrease in soil pH and a highly significant negative correlation was noted between soil pH and bacterial wilt incidence as well as pathogen population in soil (Table 4.22). Soil pH is the major contributor towards disease (91.5 %) as well as the pathogen density (65.1 %) (Fig.5.6 and 5.7). This observation is consistent with the findings of Sharma *et al.* (2010) in the bacterial wilt of ginger and Yang *et al*. (2017) in the case of wilt incidence in tomato. Furthermore, addition of bioorganic fertilizer increased the soil pH and significantly reduced the population of *R. solanacearum* in soil (Liu *et al*., 2015).

5.5.2.2 Organic carbon content

The organic carbon content in rhizosphere soil of healthy plants was found to be significantly higher as compared to diseased plants. The higher content of easily decomposable organic matter might be associated with higher microbial activity and this ultimately leads to the decline of pathogen population in soil. Lower average incidence of bacterial wilt was observed in weakly acidic soil with higher organic carbon content (Sharma *et al.,* 2010). A higher content of organic carbon was estimated

in the healthy rhizosphere soil of Marayur hills while the pathogen count was found to be comparatively lower in these soils as the population of *R. solanacearum* race 3 declines in soils having high organic matter content (Moffet *et al.,* 1983).

5.5.2.3 Available K, Ca and Fe

The role of K and Ca in imparting disease resistance to plants is well documented. Lower incidence of bacterial wilt in K and Ca rich soils was attributed to the ability of these nutrients to increase plant resistance (Messiha, 2007). In the study, both the elements were found to be significantly higher in rhizosphere soil of healthy plants as compared to diseased plants in each location. Many other workers have also studied the incidence of bacterial wilt in relation to nutrient status of soil. In all the studies, higher levels of nutrients like organic carbon, available K (Liu *et al*., 2015; Wang *et al.,* 2017) and Ca (Zheng *et al.*, 2014) has been established in the rhizosphere of healthy plants. Among the micronutrients, Fe content was significantly higher in rhizosphere soil of healthy plants. Reduced incidence of various soil borne diseases in soils rich in Fe has been reported by many workers (Duffy, 1997; Pankhurst, 2002; Rime, 2003, Janvier *et al.*, 2007).

Similar to soil pH, available Ca in soil is also negatively correlated with wilt incidence and pathogen population in rhizosphere (Table 4.22). Moreover, 76.7 per cent variation in PDI and 80.7 per cent variation in pathogen density are explained by Ca content (Fig. 5.8 and 5.9). In all the eight locations, an increased content of available Ca is associated with lower wilt incidence and pathogen population. Available Ca content in soil is positively correlated with soil suppressiveness and negatively correlated with the population of *R. solanacearum* (Zheng *et al*., 2014; Liu *et al*., 2015 and Yang *et al*., 2017)

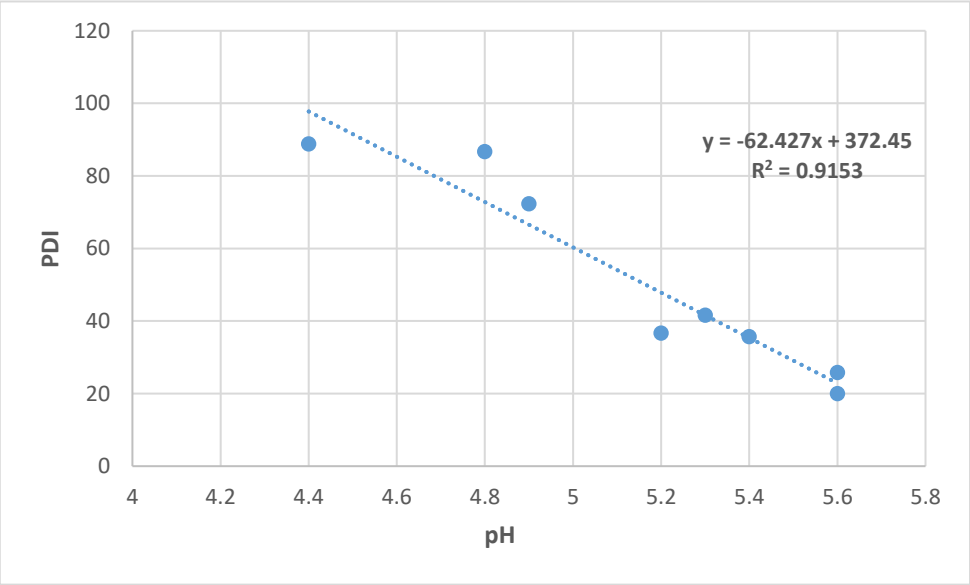


Fig. 5.6 Correlation between soil pH and PDI

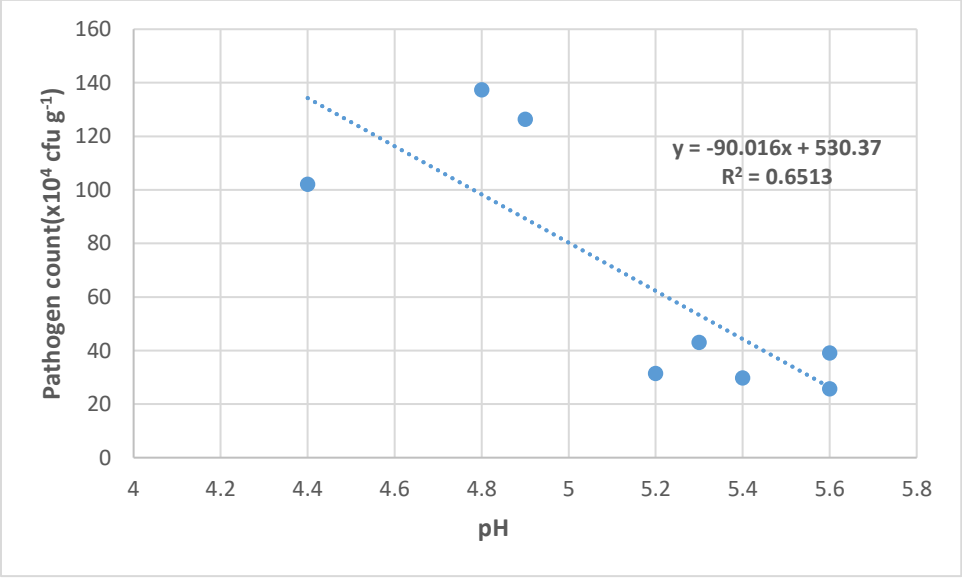


Fig. 5.7 Correlation between soil pH and population of *R. solanacearum*

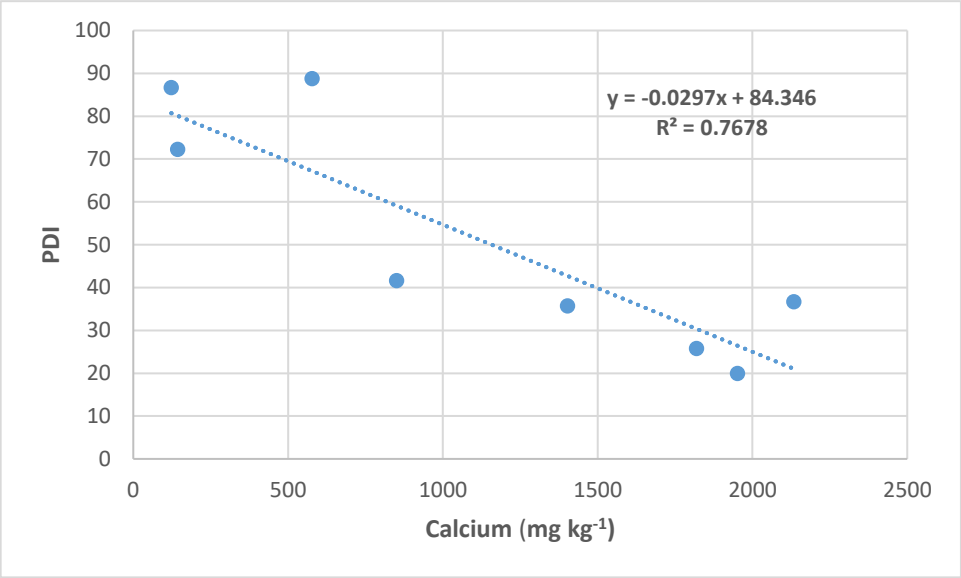


Fig. 5.8 Correlation between Available Ca and PDI

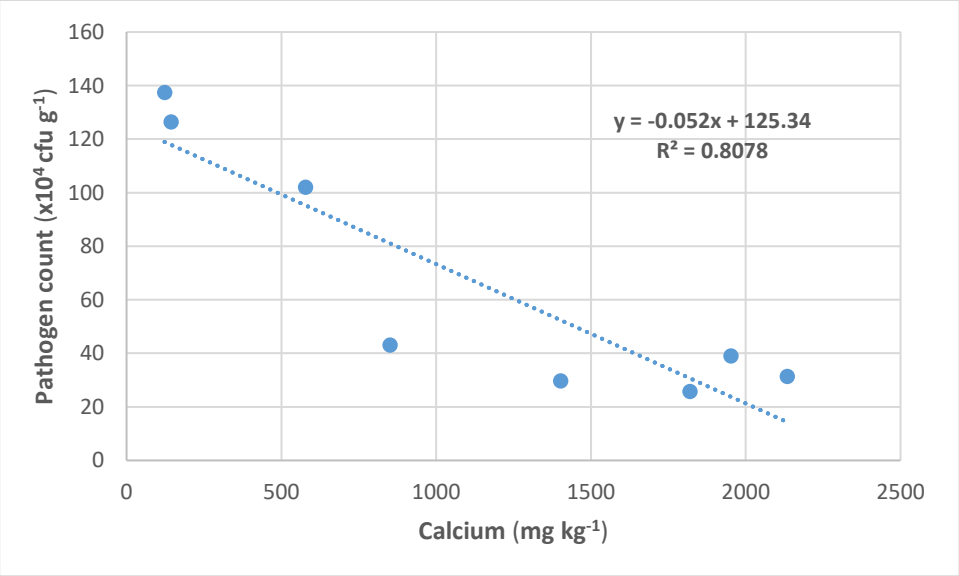


Fig. 5.9 Correlation between Available Ca and population of *R. solanacearum*

5.5.3 Enumeration of rhizosphere microflora

Identification of the biological properties contributing to soil suppressivness soils is the first step to the management and control of soil borne diseases (Mazzola, 2007). The rhizobacterial community is very important both for plant growth and plant health as it plays critical roles in regulating soil fertility, cycling of nutrients, and the protection of plants from diseases, such as the bacterial wilt (Wang *et al*. 2017). Hence, in the present investigation the population of rhizosphere microflora of healthy and diseased plants was estimated using serial dilution and plating. Despite, the fact that the overwhelming number of non culturable organisms is not considered by such techniques (Torsvik *et al.*, 1990), the number of colony forming units (cfu) on specific media are still considered informative (Balestra and Mishaghi, 1997).

In the present study, the population of fungi, bacteria, actinomycetes and pseudomonads were found to be significantly higher in rhizosphere soil of healthy plants. Soil suppressiveness brought about by soil microflora is well documented. The pathogen altered the composition of microbial communities in soil especially suppressing their functional diversity in diseased soil samples (Li *et al*., 2014). Accordingly, healthy soils exhibit higher microbial density as compared to bacterial wilt infected soil (Wang *et al.*, 2017; Qi *et al.*, 2019). The studies of Yang *et al*. (2017) also revealed that a highly diverse soil microbiome could decrease the severity of bacterial wilt outbreak in tobacco. However, in the present study the difference in population of total bacteria and fluorescent pseudomonads between the rhizosphere soil of healthy and diseased plants were more significant than fungi and actinomycetes. Hence it indicates that the competition and antagonism exerted by beneficial soil bacteria on the pathogen is obviously higher than the other soil microbial community. This can be attributed to the earlier observation that soil acidity is higher in rhizosphere soil of diseased plants which in turn favour the pathogen and inhibit beneficial antagonistic bacteria (Li *et al.*, 2017).

One of the most salient findings of this study is that, within the location there is significant difference in the population of rhizosphere microflora in healthy and diseased plants which can be attributed soil suppressiveness brought about by the beneficial microbial community. It was also found that chemical properties like soil

pH, organic carbon, available K and Ca were significantly higher in the rhizosphere soil of healthy plants. Hence it can be inferred that population of beneficial microflora is also supported by these chemical parameters. Abundance of *R. solanacearum* is observed in soils with low pH, low nutrient status and low rhizobacterial count (Zheng *et al.,* 2014). Soil pH has a strong influence on the soil microbial community structure and alkaline conditions are beneficial to the growth and activity of antagonistic microflora. Further, soil microbial balance is broken under acidic conditions leading to loss of beneficial microbes (Li *et al*., 2017). Apart from soil pH, improvement of soil fertility status by incorporation of organic matter and other soil amendments also enriched the soil microbial diversity as observed by many researchers (Li *et al*., 2009; Wu *et al.*, 2014). It is also interesting to note that same chemical parameters influence the pathogen and beneficial microflora but differently**.**

5.6 MULTIPLE REGRESSION ANALYSIS TO ASSESS THE EXTENT OF BACTERIAL WILT INCIDENCE AS INFLUENCED BY SOIL PROPERTIES

In the present investigation, soil properties like water holding capacity and bulk density showed a significant positive correlation, whereas soil pH and available Ca content in the soil exhibited a significant negative correlation with bacterial wilt incidence and pathogen population (Table 4.22). Hence an attempt was made to quantify the extent of influence exerted by these factors on disease incidence and pathogen population in soil. Oyarzun *et al.* (1998) used single correlations to attribute a statistical significance to the relation found between disease severity and each soil parameter. But this was considered insufficient as interactions between biological, physical and chemical properties of soil are of primary importance and has to be accounted (Janevier *et al*., 2007). Multiple regression analysis have been used to establish relationships between disease and the other variables and its employed when disease incidence or severity is considered as a variable that is affected by a combination of other variables. Moreover, this approach enables us to choose those variables, among all the variables measured, which are most influential in disease incidence. Considering this, soil properties which recorded high significant correlation with disease were put into step-up multiple regression analysis so as to quantify their influence on disease incidence and build-up of pathogen population in soil.

The results revealed that among the four parameters, pH and calcium content in soil emerged as most important determinants of bacterial wilt incidence of solanaceous crops which contributed significantly (96.8%) to the disease with negative correlation. A higher soil pH and Ca content in soil can result in reduced disease incidence and this can be attributed to the fact that pathogen loses virulence rapidly at high pH and soils with high pH remains healthy (Shekhawat and Perombelon, 1991). Also, soil pH determines the nutrient availability to the plants because some nutrients become "tied up" in the soil at certain pH levels. This can lead to deficiencies of calcium, magnesium, potassium and molybdenum affecting soil health and making them conducive to diseases (Fernández and Hoeft, 2009). Further, acidic conditions of soil favored the growth of the pathogen but suppressed the growth and activity of antagonistic bacteria (Li *et al.*, 2017). As mentioned earlier, pH of soil exerts an influence on Ca content in soil and an increase in Ca nutrition to near the level of physiological sufficiency of plants significantly reduced the severity of wilt as observed by He *et al*. (2014). These suggest that regulation of soil acidification is the precondition and foundation of controlling bacterial wilt.

Earlier results reveal that the population of pathogen in soil is influenced by soil physico-chemical parameters. Step-up multiple regression analysis indicates that bulk density and Ca content contributed 92.2 per cent in the build-up of pathogen population in soil. Content of Ca^{2+} is found to have a direct correlation with the suppressive effect on the pathogen. With increase in Ca concentration, the pectinase activity of *R. solanacearum* decreased significantly thus delaying the entrance of the bacteria to the host plant (He *et al*., 2014). This might be the reason which holds available Ca in the soil as one of the major factor determining the incidence of bacterial wilt as well as build-up of pathogen population in soil. Further, soils with low bulk density supports the growth and multiplication of beneficial microflora (Li *et al.*, 2017) which in turn suppresses the pathogen and hence the disease. Also, the availability of various nutrients especially Ca in soil is negatively correlated to bulk density which further predisposes the plants to bacterial wilt (Chaudari *et al*., 2013).

Utilizing the results of multiple regression analysis prediction equations were developed to assess the per cent disease incidence as well as pathogen population in soil. Only very limited number of researchers have attempted to deduce such equations

for bacterial wilt. Sharma *et al*. (2010) has developed a prediction equation for bacterial wilt of ginger in which soil pH and organic carbon were the major determinants.

The purposive survey undertaken in four different agro ecological units for bacterial wilt incidence indicated that the variations in wilt incidence can be due to the variability in the population of *R. solanacearum* in soil as well as the different soil factors prevailing in different locations surveyed. Further, the characterisation of *R. solanacearum* isolates collected from these locations suggested distinct variability of the pathogen. This is due to the difference in soil properties in these locations as evidenced by the fact that, isolates from same agro ecological units are more phylogenetically related. Also, the soil pH, organic carbon, available K, Ca and Fe content in soil were found to be significantly higher in rhizosphere soil of healthy plants. There was significantly more number of beneficial microflora in the rhizosphere of healthy plants. Moreover, multiple regression analysis indicated that soil pH and available Ca are the most influential parameters contributing to wilt incidence whereas soil bulk density and Ca content significantly influenced the pathogen population. Hence, based on the salient findings of the study it may be concluded that, manipulation of soil factors can play a major role in integrated management of the disease. Furthermore, since soil factors play a role in the variability of pathogen, this may be considered while planning resistance breeding programmes against bacterial wilt caused by *R. solanacearum*.

6. Summary

6. SUMMARY

An investigation on 'Characterisation of *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. infecting solanaceous vegetables in relation to physico-chemical and biological properties of soil' was carried out at Department of Plant Pathology, College of Horticulture, Vellanikkara, Kerala. The objective of the study was to isolate and characterise *Ralstonia solanacearum* infecting solanaceous vegetables from four different agro ecological units of Kerala and to study the soil physico-chemical and biological properties influencing the pathogen. The prominent findings of the study are outlined below.

- A purposive sampling survey was conducted in four agro ecological units (AEUs) of Kerala *viz.* North Central laterite (NCP), Marayur hills (MH), Southern laterite (SL) and Palakkad central plains (PCP). The wilted plant samples along with the rhizosphere soil of both healthy and infected plants were collected. Two locations from each of these four AEUs were surveyed for wilt incidence from March to November 2018.
- The per cent disease incidence (PDI) of the locations was recorded and varied from 88 per cent in the location NCL 1 (Pudukkad) to 20 per cent in the location PCP 1 (Vithanassery). The locations SL 1 (Pravachambalam) and SL 2 (Venganoor) recorded a high PDI of 72.3 per cent and 86.7 per cent respectively. The two locations of Marayur hills MH 1 (Perumalai) and MH 2 (Kanthaloor) exhibited a comparatively lower wilt incidence, that is, 35.7 per cent and 25.8 per cent respectively.
- The bacterium associated with infected plants was isolated on TZC agar and the pathogenicity was proved. Thus, a total of eight isolates of the pathogen were collected and maintained for all the experiments.
- The population of the pathogen present in the rhizosphere soil ranged from 20.66 x 10^4 cfu $g^{-1}$of rhizosphere soil of healthy plants of MH 2 to 137.66 x 10^4 cfu g^{-1} of rhizosphere soil of diseased plants of SL 2. A significant difference in pathogen population was observed between the rhizosphere soil of diseased and healthy plants of the same location.

- A positive correlation was observed between the PDI and pathogen population in different locations and 84.8 per cent of variation bacterial wilt incidence is explained by the population of pathogen.
- All the isolates produced typical circular colonies which are creamy white with faint pink to reddish center and with entire margin, but variations were observed in the case of size, pigmentation, elevation and fluidity. The size of the colonies ranged from 1.5 mm to 5.5 mm.
- Morphological characterisation of the bacterium revealed Gram negative rod shaped cells measuring 0.3-0.5 µm x 1.2-1.7 µm under scanning electron microscope.
- *In-silico* analysis of 16S rRNA sequences confirmed the identity of all isolates as *Ralstonia solanacearum* (Smith) Yabuuchi *et al.*
- Phylogenetic analysis revealed that the eight isolates collected from different AEUs clustered on different branches of the phylogenetic tree while those from the same AEUs clustered together. This indicates considerable variation among the isolates in accordance with location.
- Pathogenicity on differential hosts identified the isolates from Marayur hills as race 3 and all the other isolates as race 1.
- Based on utilisation of disaccharides and hexose alcohols the isolates from NCL and SL were classified as biovar III, strains from PCP as biovar III A and those of MH as biovar II.
- Soil temperature in the range of 25.2°C to 37.8°C and clay loam soil which are conducive to bacterial wilt was observed in all the locations. Bulk density fell within the optimum limit of plant growth, ranging from 1.04 to 1.29 g cm^{-3} and water holding capacity varied from 38.66 per cent to 51.27 per cent. Statistical analysis using paired sample t-test revealed no significant difference in physical properties of rhizosphere soil of healthy and diseased plants within the location.
- The soil reaction was predominantly acidic in all the locations, pH ranging from 4.4 to 6.5. Organic carbon content ranged from 0.55 % to 2.39 % whereas available P varied from 67.31 kg ha^{-1} to 600.78 kg ha^{-1}. The available K and Ca content fluctuated from 2321.76 kg ha^{-1} to 71.38 kg ha^{-1} and 2220.64 mg kg^{-1} to 122.76 mg kg^{-1} respectively. Similarly, Fe and Mn ranged from 156.2 mg kg^{-1} to

14.63 mg kg-^{1} and 19.25 mg kg^{-1} to 256.60mg kg^{-1} respectively. Soil pH, organic carbon content, available K, available Ca and Fe were significantly higher in the rhizosphere soil of healthy plants compared to diseased plants.

- Significantly higher population of rhizosphere microflora (fungi, total bacteria, actinomycetes and fluorescent Pseudomonads) was observed in the case of healthy plants. However, the difference in population of bacteria and fluorescent pseudomonads between healthy and diseased plants was more predominant.
- Water holding capacity as well as bulk density of rhizosphere soil was found to be positively correlated with PDI and population of pathogen in soil whereas soil pH and available Ca were negatively correlated.
- Step-wise multiple regression analysis indicated that 96.8 per cent variation in the bacterial wilt incidence is explained by soil pH and available Ca content in the rhizosphere soil with negative correlation whereas bulk density and Ca content contributed 92.2 per cent in the build-up of population of *Ralstonia solanacearum* in soil.

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Appendices

APPENDIX – I

A. Composition of media

1. Triphenyl tetrazolium chloride agar (TZC)

Peptone	-	10 g
Casein hydrolysate	-	1 g
Glucose	-	5.0 g
Agar	-	20.0 g
Distilled water	-	1000 ml
pH	-	7

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

2. Casaminoacid peptone glucose agar (CPG)

Peptone	-	10 g
Casein hydrolysate	-	1 g
Glucose	-	5.0 g
Agar	-	20.0 g
Distilled water	-	1000 ml
pH	-	7

3. Selective medium South Africa (SMSA)

Casein hydrolysate	-	1 g
Glycerol	-	5 ml
Peptone	-	10 g
Agar	-	15 g
Bacitracin	-	25 mg
Chloramphenicol	-	5 mg
Crystal violet	-	5 mg
Cycloheximide	-	100 mg
Penicillin G	-	0.5 mg
Polymyxin sulfate	-	100 mg
TZC	-	50 mg
Distilled water	-	1000 ml

4. Martin's rose bengal streptomycin agar (MRBSA)

Dextrose	-	10.0 g
Peptone	-	5.0 g
KH_2PO_4	-	1.0 g
$MgSO_4$	-	0.5 g
Agar	-	20.0 g
Rose Bengal	-	0.03 g
Streptomycin	-	30.0 mg
Distilled water	-	1000 ml

5. Nutrient agar (NA)

Peptone	-	20.0 g
Beef extract	-	1.0 g
NaCl	-	5.0 g
Agar	-	20 g
Distilled water	-	1000 ml
pH	-	7.2

6. Kenknights agar

Dextrose	-	1.0 g
KH_2PO_4	-	0.1 g
$NaNO_3$	-	0.1 g
KCl	-	0.1 g
$MgSO_4$	-	0.1 g
Agar	-	20.0 g
Distilled water	-	1000 ml
pH	-	7

7. King's B agar (KB)

Peptone	-	20.0 g
Glycerol	-	10 ml

K_2HPO_4	-	1.5 g
$MgSO_4.7H_2O$	-	1.5 g
Agar	-	20.0 g
Distilled water	-	1000 ml
pH	-	7.2

8. Casaminoacid peptone glucose broth

Peptone	-	10 g
Casein hydrolysate	-	1 g
Glucose	-	5.0 g
Distilled water	-	1000 ml
pH	-	7

9. Hayward's basal medium

Peptone	-	1.0 g
$NH_4H_2P\ 0_4$	-	1.0 g
KCI	-	0.2 g
$MgS0_4\ .2H_20$	-	0.2 g
Bromothymol blue	-	0.08 g
Distilled water	-	1000 ml
pH	-	7.5

APPENDIX – II

I. Reagents for DNA isolation

A. TE Buffer

(Tris HCl – 10.0 mM; EDTA – 1.0 mM)

Prepare 1000 ml using

100 X TE Tris, pH 8.0 - 10 ml

Distilled water - 990 ml

B. Sodium dodecyl sulphate (10 %)

Sodium dodecyl sulphate - 10 g

Distilled water - 100 ml

C. Phenol-Chloroform (1: 2 v/v)

To one part of phenol, one part of chloroform added and mixed properly.

D. Ethanol (70%)

To seventy parts of absolute ethanol thirty parts of distilled water was added and mixed well.

II. Composition of buffers and dyes used for gel electrophoresis

A. TAE buffer (50X)

Tris base - 242 g

Glacial acetic acid - 57.1 ml

0.5 M EDTA (pH-8) - 100 ml

B. Loading dye (6X)

Bromophenol blue - 0.25 %

Xylene cyanol - 0.25 %

Glycerol in water - 30.0 %

APPENDIX – III

I. Nucleotide sequence of 16S rDNA of *R. solanacearum* isolates

NCL 1

5’CCGCGATTACTAGCGATTCCAGCTTCACGTAGTCGAGTTGCAGACTACGATCCACTACGATGC
ATTTTCTGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTCTGTATGCACCATTGTATGACGTGT
GAAGCCCTACCCATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACC
GGCAGTCTCTCTAGAGTGCCCTTTCGTAGCAACTAGAGACAAGGGTTGCGCTCGTTGCGGGACT
TAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCCACTTTCTCTTTC
GAGCACCTAATGCATCTCTGCTTCGTTAGTGGCATGTCAAGGGTAGGTAAGGTTTTTCGCGTTGC
ATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATCT
TGCGACCGTACTCCCCAGGCGGTCAACTTCACGCGTTAGCTACGTTACTAAGGAAATGAATCCC
CAACAACTAGTTGACATCGTTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCA
CGCTTTCGTGCATGAGCGTCAGTGTTATCCCAGGAGGCTGCCTTCGCCATCGGTATTCCTCCACA
TCTCTACGCATTTCACTGCTACACGTGGAATTCTACCTCCCTCTGACACACTCTAGCCGTGCAGT
CACCAATGCAATTCCCAGGTTAAGCCCGGGGATTTCACATCGGTCTTGCACAACCGCCTGCGCA
CGCTTTACGCCCAGTAATTCCGATTAACGCTTGGACCCTACGTATTACCGCGGCTGCTGGCACGT
AGTTAGCCGGTCCTTATTCTTCCGGTACCGTCATCCACACCAGGTATTAACCAGTGCGATTTCTT
TCCGGACAAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATTGCTGGATCAGGG
TTGCCCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCC
CAGTGTGGCTGATCGTCCTCTCAGACCAGCTACTGATCGTCGCCTTGGTGGGCCTTTACCCCACC
AACTAGCTAATCAGACATCGGCCGCTCCTATAGCATGAGGCCTTGCGGTCCCCCACTTTCACCCT
CAGGTCGTATGCGGTAGCTAGTCTTTCGACTAGTTATCCCCCACTACAGGGCACGTTCCGATGTA
TTACTCACCCGTTCGCCA3’

NCL 2

5’ACGGGTGAGTAATACATCGGAACGTGCCCTGTAGTGGGGGATAACTAGTCGAAAGATTA
GCTAATACCGCATACGACCTGAGGGTGAAAGTGGGGGACCGCAAGGCCTCATGCTATAGGAGCG
GCCGATGTCTGATTAGCTAGTTGGTGGGGTAAAGGCCCACCAAGGCGACGATCAGTAGCTGGTC
TGAGAGGACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTC
GGGTTGTAAAGCACTTTTGTCCGAAAGAAATGGCTCTGGTTAATACCTGGGGTCGATGACGGTAC
CGGAAGAATAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCCAAGCGTT
AATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTGTGCAAGACCGATGTGAAATCCCCGA
GCTTAACTTGGGAATTGCATTGGTGACTGCACGGCTAGAGTGTGTCAGAGGGGGGTAGAATTCC
ACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGG
ATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCCTAAACGATGTCAACTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAGCTAACGCGTGAA
GTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACA
AGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAC
TAACGAAGCAGAGATGCATTAGGTGCTCGAAAGAGAAAGTGGACACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTTAGTTGCT
ACGAAAGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAG
TCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTGCATACAGAGGGTTGCCAAG
CCGCGAGGTGGAGCTAATCCCAGAAAATGCATCGTAGTCCGGATCGTAGTCTGCAACTCGACTA
CGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTG
TACACACCGCCCGTCACACCATGGGAGT 3

MH 1

5’TGCCCTGTAGTGGGGGATAACTAGTCGAAAGACTAGCTAATACCGCATACGACCTGAGG
GTGAAAGTGGGGGACCGCAAGGCCTCATGCTATAGGAGCGGCCGATGTCTGATTAGCTAGTTGG
TGGGGTAAAGGCCCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGATCAGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGCA
ACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGA
AAGAAATCGCACTGGTTAATACCTGGTGTGGATGACGGTACCGGAAGAATAAGGACCGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGGTCCAAGCGTTAATCGGAATTACTGGGCGTAAAG
CGTGCGCAGGCGGTTGTGCAAGACCGATGTGAAATCCCCGGGCTTAACCTGGGAATTGCATTGG
TGACTGCACGGCTAGAGTGTGTCAGAGGGAGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAG
AGATGTGGAGGAATACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCACGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTT
GTTGGGGATTCATTTCCTTAGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTC
GCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATT
CGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCACTAACGAAGCAGAGATGCATTAGG
TGCTCGAAAGAGAAAGTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTCTAGTTGCTACGAAAGGGCACTCTAGAGAG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAG
GGCTTCACACGTCATACAATGGTGCATACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCC
AGAAAATGCATCGTAGTCCGGATCGTAGTCTGCAACTCGACTACGTGAAGCTGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACGGTAGGGTTCATGA
CTGG 3’

MH 2

5’CAGACTTCACGTAGTCGAGTTGCAGACTACGGATCCGGACTACGATGCATTTTCTGGGA
TTAGCTCCACCTCGCGGCTTGGCAACCCTCTGTATGCACCATTGTATGACGTGTGAAGCCCTACC
CATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCTCT
AGAGTGCCCTTTCGTAGCAACTAGAGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCT
CACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCCACTTTCTCTTTCGAGCACCTAATGC
ATCTCTGCTTCGTTAGTGGCATGTCAAGGGTAGGTAAGGTTTTTCGCGTTGCATCGAATTAATCC
ACATCATCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCC
CCAGGCGGTCAACTTCACGCGTTAGCTACGTTACTAAGGAAATGAATCCCCAACAACTAGTTGAC
ATCGTTTAGGGCGTGGACTACCAGGGCTGCCTTCGCCATCGGTATTCCTCCACATCTCTACGCATT
TCACTGCTACACGTGGGAATTCTACCTCCCTCTGACACACTCTAGCCGTGCAGTCACCAATGCAA
TTCCCAGGTTAAGCCCGGGGGATTTCACATCGGTCTTGCACAACCGCCCTGCGCACGCTTTACGC
CCAGTAATTCCGATTAACGCTTGGACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGG
TCCTTATTCTTCCGGTACCGTCATCCACACCAGGTATTAACCAGTGCGATTTCTTTCCGGACAAAA
GTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATTGCTGGATCAGGGTTGCCCCCATTG
TCCAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGA
TCGTCCTCTCAGACCAGCTACTGATCGTCGCCTTGGTGGGCCTTTACCCCACCAACTAGCTAATC
AGACATCGGCCGCTCCTATAGCATGAGGCCTTGCGGTCCCCCACTTTCACCCTCAGGTCGTATGC
GGTATTAGCTAGTCTTTCGACTAGTTATCCCCCACTACAGGGCACGTTCCGATGTATTA 3’

SL 1

5’CCGCGATTACTAGCGATTCCAGCTTCACGTAGTCGAGTTGCAGACTACGATCCGGACTA
CGATGCATTTTCTGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTCTGTATGCACCATTGTATGA
CGTGTGAAGCCCTACCCATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGTTTGT
CACCGGCAGTCTCTCTAGAGTGCCCTTTCGTAGCAACTAGAGACAAGGGTTGCGCTCGTTGCGGG
ACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCCACTTTCTCT
TTCGAGCACCTAATGCATCTCTGCTTCGTTAGTGGCATGTCAAGGGTAGGTAAGGTTTTTCGCGTT
GCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATC
TTGCGACCGTACTCCCCAGGCGGTCAACTTCACGCGTTAGCTACGTTACTAAGGAAATGAATCCC
CCAACAACTAGTTGACATCGTTTAGGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCC
ACGCTTTCGTGCATGAGCGTCAGTGTTATCCCAGGAGGCTGCCTTCGCCATCGGTATTCCTCCAC
ATCTCTACGCATTTCACTGCTACACGTGGAATTCTACCTCCCTCTGACACACTCTAGCCGTGCAGT
CACCAATGCAATTCCCAGGTTAAGCCCGGGGATTTCACATCGGTCTTGCACAACCGCCTGCGCAC
GC5TTTACGCCCAGTAATTCCGATTAACGCTTGGACCCTACGTATTACCGCGGCTGCTGGCACGT
AGTTAGCCGGTCCTTATTCTTCCGGTACCGTCATCCACACCAGGTATTAACCAGTGCGATTTCTTT
CCGGACAAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATTGCTGGATCAGGGTT
GCCCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA
GTGTGGCTGATCGTCCTCTCAGACCAGCTACTGATCGTCGCCTTGGTGGGCCTTTACCCCACCAA
CTAGCTAATCAGACATCGGCCGCTCCTATAGCATGAGCCTTGCGGTCCCCCACTTTCACCCTCAG
GTCGTATGCGGTATTAGCTAGTCTTTCGACTAGTTATCCCCCACTACAGGGCACGTTCCGATGTAT
TACTCACCCGTTCGCCA 3’

SL 2

5’ACTAGCGATTCCAGCTTCACGTAGTCGAGTTGCAGACTACGATCCGGACTACGATGCAT
TTTCTGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTCTGTATGCACCATTGTATGACGTGTGA
AGCCCTACCCATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGC
AGTCTCTCTAGAGTGCCCTTTCGTAGCAACTAGAGACAAGGGTTGCGCTCGTTGCGGGACTTAAC
CCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCCACTTTCTCTTTCGAGC
ACCTAATGCATCTCTGCTTCGTTAGTGGCATGTCAAGGGTAGGTAAGGTTTTTCGCGTTGCATCG
AATTAATCCACATCATCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGA
CCGTACTCCCCAGGCGGTCAACTTCACGCGTTAGCTACGTTACTAAGGAAATGAATCCCCAACAA
CTAGTTGACATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCG
TGCATGAGCGTCAGTGTTATCCCAGGAGGCTGCCTTCGCCATCGGTATTCCTCCACATCTCTACG
CATTTCACTGCTACACGTGGAATTCTACCTCCCTCTGACACACTCTAGCCGTGCAGTCACCAATG
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CCCAGTAATTCCGATTAACGCTTGGACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCG
GTCCTTATTCTTCCGGTACCGTCATCCACACCAGGTATTAACCAGTGCGATTTCTTTCCGGACAAA
AGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATTGCTGGATCAGGGTTGCCCCCATT
GTCCAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTG
ATCGTCCTCTCAGACCAGCTACTGATCGTCGCCTTGGTGGGCCTTTACCCCACCAACTAGCTAAT
CAGACATCGGCCGCTCCTATAGCATGAGGCCTTGCGGCCCCCACTTTCACCCTCAGGTCGTATGC
GGTATTAGCTAGTCTTTCGACTAGTTATCCCCCACTACAGGGCACGTTCCGATGTATTACTCACCC
GTTCGCCAC 3’

PCP 1

5’TGCAGTCGAACGGCAGCGGGGGTAGCTTGCTACCTGCCGGCGAGTGGCGAACGGGTGA
GTAATACATCGGAACGTGCCCTGTAGTGGGGGATAACTAGTCGAAAGACTAGCTAATACCGCAT
ACGACCTGAGGGTGAAAGTGGGGGACCGCAAGGCCTCATGCTATAGGAGCGGCCGATGTCTGAT
TAGCTAGTTGGTGGGGTAAAGGCCCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGATC
AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGAC
AATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCAC
TTTTGTCCGGAAAGAAATCGCACTGGTTAATACCTGGTGTGGATGACGGTACCGGAAGAATAAG
GACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCCAAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGTGCGCAGGCGGTTGTGCAAGACCGATGTGAAATCCCCGGGCTTAACCTGGG
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GAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGC
TCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGAT
GTCAACTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGG
GGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGA
TGTGGATTAATTCGATGCAACGCGAAAAACCTACCTACCCTTGACATGCCACTAACGAAGCAGA
GATGCATTAGGTGCTCGAAAGAGAAAGTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTCTAGTTGCTACGAAGGGCAC
TCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCT
TATGGGTAGGGCTTCACACGTCATACAATGGTGCATACAGAGGGTTGCCAAGCCGCGAGGTGGA
GCTAATCCCAGAAAATGCATGTAGTCCGGATCGTAGTCTGCAACTCGACTACGTGAAGCTGGAA
TCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTG 3’

PCP 2

5’AGGCCGGGCTGCCTTACACATGCAAGTCGAACGGCAGCGGGGGTAGCTTGCTACCTGCCGGC
GAGTGGCGAACGGGTGAGTAATACATCGGAACGTGCCCTGTAGTGGGGGATAACTAGTCGAAA
GACTAGCTAATACCGCATACGACCTGAGGGTGAAAGTGGGGGACCGCAAGGCCTCATGCTATA
GGAGCGGCCGATGTCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTA
GCTGGTCTGAGAGGACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATTTTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGA
AGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCGCACTGGTTAATACCTGGAGTGG
ATGACGGTACCGGAAGAATAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GTCCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTGTGCAAGACCGATGT
GAAATCCCCGGGCTTAACCTGGGAATTGCATTGGTGACTGCACGGCTAGAGGTGTCAGAGGGA
GGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGC
AGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAG
CTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGAC
GGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTA
CCCTTGACATGCCACTAACGAAGCAGAGATGCATTAGGTGCTCGAAAGAGAAAGTGGACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGTCTCTAGTTGCTACGAAAGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTGC
ATACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCAGAAAATGCATCGTAGTCCGGATC
GTAGTCTGCAACTCGACTACGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGG3’

CHARACTERISATION OF *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* INFECTING SOLANACEOUS VEGETABLES IN RELATION TO PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF SOIL

By

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(2017-11-015)

ABSTRACT OF A THESIS

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ABSTRACT

Ralstonia solanacearum, the causal agent of vascular wilt disease of crop plants has been ranked as the second most important bacterial pathogen in the world next to *Pseudomonas syringae*. The high diversity exhibited by the pathogen hampers the breeding for resistance, consequently the resistant varieties developed may not express uniform level of resistance in different geographical locations. Being a soil inhabitant, the survival of *R. solanacearum* is influenced by physico-chemical and biological properties of soil. Considering these facts, present investigation was carried out with the objective of characterisation of *R. solanacearum* from different agro ecological units (AEUs) of Kerala and to determine the effect of soil physical, chemical and biological properties on the pathogen.

Purposive sampling survey was conducted in four AEUs of Kerala *viz.*, North Central laterite (NCL), Marayur hills (MH), Southern laterite (SL) and Palakkad central plains (PCP) from March to September 2018. Two locations from each AEUs were selected for the survey. The per cent incidence in different locations ranged from 20 to 88 per cent. The pathogen was isolated from infected plant samples collected during the survey on triphenyl tetrazolium chloride (TZC) agar and pathogenicity was proved by inoculation on respective hosts. The rhizosphere soil samples of healthy and diseased plants were collected from each location. The population of the pathogen present in the soil was quantified and it ranged from 20.66 x 10^4 cfu $g^{-1}$of soil to 137.66 x 10^4 cfu g^{-1} of soil. A strong positive correlation was observed between pathogen density in soil and per cent disease incidence (PDI).

A total of eight isolates were collected, purified and maintained for all the experiments. The colony characters of different isolates on TZC agar showed considerable variation and the morphology of the bacterial cells was studied using scanning electron microscopy. Typical rod shaped cells with size 0.3-0.5 µm x 1.2-1.7 µm were observed. The molecular characterization of the isolates was done by PCR amplification and sequencing of 16S rRNA. The sequences were subjected to *in-silico* analysis which confirmed the identity of all isolates as *R. solanacearum* (Smith) Yabuuchi *et al.* The phylogenetic analysis revealed that the eight isolates collected from different AEUs clustered on different branches of the tree while those from the

same AEUs clustered together. This indicates considerable variation among the isolates in accordance with location which can be attributed to the difference soil parameters in these locations.

The isolates of the pathogen were further categorized into races and biovars based on pathogenicity on differential hosts and utilization of disaccharides and hexose-alcohols respectively. The results revealed that two isolates from Marayur hills (MH 1 and MH 2) belong to race 3, biovar II whereas two from Palakkad central plains (PCP 1 and PCP 2) belong to race 1 biovar III A. The other four isolates collected from Northern central laterite (NCL 1 and NCL 2) and Southern laterite (SL 1 and SL 2) were identified as race 1, biovar III.

The physico-chemical and biological properties of the soil samples collected during the survey were analysed using standard protocols. The statistical analysis using paired sample t-test revealed significantly higher soil pH, organic carbon, available K, Ca and Fe content and soil microflora in rhizosphere soil of healthy plant compared to diseased. A significant positive correlation was observed between PDI and soil parameters *viz*., water holding capacity and bulk density whereas soil pH and available Ca content exhibited a negative correlation with PDI. A similar trend was observed in the case of pathogen population also. Further, multiple regression analysis was performed to assess the extent of variation contributed by different soil parameters on PDI and pathogen population. The results indicate that 96.8 per cent variation in the bacterial wilt incidence is explained by soil pH and available Ca content in the rhizosphere soil with negative correlation whereas bulk density and Ca content contributed 92.2 per cent in the build-up of population of *Ralstonia solanacearum* in soil.

The study revealed the influence of soil factors on bacterial wilt disease incidence, population of *R. solanacearum* and pathogen variability. Hence, it is concluded that, manipulation of soil factors can play a major role in integrated management of the disease. Furthermore, the variability of pathogen according to geographical region, may be considered while planning resistance breeding programmes against bacterial wilt disease.