2
Diagnosis

Detection and identification of bacterial pathogen causing bacterial wilt is included in diagnosis. In diagnosis, it tries to prove that a bacterium isolated from a diseased plant, and identified, using different techniques, is really the cause of the disease observed. There are various steps involved in a diagnosis process, which are actually Koch's postulates. The steps are assessment of symptoms, isolation of pathogenic bacteria, growing pure culture of isolated bacteria, identification of pure culture, pathogenicity test, reisolation from inoculated plants, reidentification of reisolate and diagnosis report.

2.1 SYMTOMATOLOGY

2.1.1 Tomato

The wilting symptom of tomato can be induced by bacterial and fungal pathogen, root-knot nematode (Libman and Leach, 1962; Libman *et al.*, 1964; Nirmala Devi and Tikoo, 1992) and deficit or excess of soil moisture (Jone *et al.,* 1991). The most characteristics symptoms of tomato are very rapid witlings, especially where the plants are young and succulent. The flowering stage is the most critical stage where a plant shows sudden wilting (Gowda *et al.*, 1974; Sonada, 1975). Other primary indications of infection are stunting, downward curling of leaflets and petioles and, where pathogenesis is slow, excessive production of adventitious roots along the stem. Usually infected plants collapse quickly, but where this fails to happen there is a development of blackening of vascular system at the junction between stem and leaf. Further down the stem, the whole vascular system may be completely blackened and when cut oozes creamy bacterial slime. Buddenhagen and Kelman (1964) have summarized the syndrome of bacterial wilt in tomato. General symptoms of bacterial wilt have been shown in Fig. 2.1 and summarised in Table 2.1.

The first visible symptom is the flaccid appearance of the youngest leaves and in the field, this symptom does not happen until after flowering has begun (McCarter, 1991). Under favourable environmental conditions for the pathogen (soil temperatures of approximately 25°C; saturated humidity), epinasty and wilting of one side or of the whole plant follows within a few days leading to total plant collapse. Under less favourable conditions (soil temperature below 21°C), less wilting occurs, but large numbers of adventitious roots may develop on the stem. It is possible to observe water soaked streaks from the base of the stem which is evidence of necrosis in the vascular system. When the stem is cut crosswise, discoloured brown vascular tissues exude white or yellowish bacterial ooze.

2.1.2 Brinjal and Chilli

Sudden wilting of foliage of the youngest leaves, which may occur during night, is the initial symptom of the wilt disease (Fig. 2.1b, c). The disease progresses with permanent wilting of entire plant, with slight or no leaf yellowing. Infected young plants die immediately, while older plants may first show leaf drooping and discolouration only one side or part of the plant and eventually the whole plant wilts and dies. The vascular system in affected plants is discoloured from pale yellow to brown in colour. A simple diagnostic test is to place cross section of the infected tissue suspended in water that yields milky white exudates of the bacterial cells.

2.1.3 Potato

The early stage of infection in the field is recognized by wilting of the leaves towards the top of the plant at high temperatures during the day with recovery at night (Fig. 2.1d). In early stages of wilting leaves remain green, but later yellowing and brown necrosis develops. Epinasty also occurs in potato plants. Wilting of one shoot or whole plants becomes rapidly irreversible and results in the collapse and death of the plant. The vascular tissue of transversely cut stems from wilted plants usually appears brown and milky bacterial ooze exudes from the cut surface or can be expressed by squeezing. When a cut stem is placed vertically in water, threads of slime will stream from the vascular bundles. Potato tubers must be cut transversely close to the heel (stolon end) or longitudinally over the stolon end. Tubers may show bacterial ooze on the eyes and stolon end attachment. Soil sticks to the tubers at the eyes. The early stage of infection is recognized by a glassy yellow to light brown discolouration of the vascular ring from which a pale cream bacterial ooze emerges spontaneously after some minutes. Later, the vascular discolouration becomes a more distinct brown and necrosis can extend into the parenchymatous tissue. In advanced stages, infection breaks outwards from the heel end and the eyes from which bacterial slime may ooze causing soil particles to adhere. Reddish-brown slightly sunken lesions may appear on the skin due to collapse of vascular tissues internally. Symptoms may be confused with those of ring rot, caused by *Clavibacter michiganensis* subsp. *sepedonicus*. Wilting and other foliage symptoms may not occur under dry and cool conditions. Secondary development of fungal and bacterial soft rots is common in the advanced stages of the disease. Fucikovsky (1980) developed methods for diagnosing *P. solanacearum* of potato bacterial disease.

2.1.4 Other Hosts

Wilting symptoms are rarely observed in natural conditions on weed hosts *viz*; *Solanum dulcamara* and *S. nigrum* unless soil temperatures exceed 25°C or inoculum levels are extremely high (e.g. as for *S. nigrum* growing adjacent to diseased potato or tomato plants). When wilting does occur, the symptoms are as described for tomato. Non-wilting *S. dulcamara* plants growing with stems and roots in water may show internal light brown discolouration of vascular tissues on transverse section of the stem base or underwater stem parts. Bacteria may ooze from cut vascular tissues or form threads of slime if the cut stem is placed vertically in water, even in the absence of wilting symptoms.

Table 2.1 Wilt disease syndrome in tomato *(R. solanacearum.)* in relation to mechanisms of pathogenesis

Symptoms	*Cause*
External symptoms	
Foliage wilting	Interference with water movement by formation of extra cellular polysaccharides, bacterial cells and tyloses in the vessels and tracheids
Foliage yellowing	Breakdown of chlorophyll resulting from decreased supply of mineral nutrients and water, unknown effects to host and pathogen metabolites
Marginal leaf necrosis	Decreased supply of water and unknown factors
Leaf epinasty	Increased level of indole-acetic acid (IAA) and ethylene (formed by pathogen or host).
Adventitious roots	Increase in IAA, interference with downward movement in phloem resulting from effects of pathogen.
Stunting	Complex of above.
Internal Symptoms	
Vascular discolouration	Tyrosinase of pathogen
Tyloses Vessel Collapse Parenchyma proliferation	Increase in IAA levels.
Dissolution of pectic substances in middle lamella	Pectin methyl esterase, olygalacturonase
Degradation of cellulose on cell walls.	Cellulose.

Agrios (2005)

2.2 BACTERIAL OOZE

Ralstonia solanacearum is a limited xylem-invading pathogen and plants wilted by *R. solanacearum* have > 10^8 cfu/g of tissue. A common sign of bacterial wilt of tomato observed at the surface of freshly-cut sections from severely infected stems is a sticky, milky-white exudates, which indicates the presence of dense masses of bacterial cells in infected vascular bundles, and particularly in the xylem, which is responsible for transportation of raw sap (water and nutrients) from roots to aerial parts of the plant. Ooze also may accumulate on the cut surface (Fig. 2.2). Allen *et al.* (2001) reported that even if ooze does not form spontaneously a streaming test may be positive. Other wilt inducing pathogens do not produce comparable ooze. The ooze is usually an almost pure culture of *R. solanacearum,* which can be cultured on standard low ionic strength bacteriological media. This water streaming test (Ooze test) is of presumptive diagnostic value in the field.

2.3 ISOLATION OF *RALSTONIA SOLANACEARUM* FROM WILTED PLANTS

(a) Remove ooze or sections of discoloured tissue from the vascular ring in the potato tuber or from the vascular strands in stems of potato, tomato or other wilting host plants. Suspend in a small volume of sterile distilled water or 50 mM phosphate buffer and leave for 5 to 10 minutes.
(b) Prepare a series of decimal dilutions of the suspension.

Fig. 2.2 Bacterial ooze exudating out from the end

(c) Transfer 50-100 µl of the suspension and dilutions to a general medium (CPG) and/or to Kelman's tetrazolium medium and/or a validated selective medium (e.g. TTC, SMSA). Spread or streak with an appropriate dilution plating technique. If useful, prepare separate plates with a diluted cell suspension of *R. solanacearum* biovar 2 as a positive control.

(d) Incubate the plates for two to six days at 28°C.

2.4 MEDIA USED FOR ISOLATION

2.4.1 Non-selective Media

These media can also be used for isolation of *R. solanacearum* from fresh, symptomatic plants, due to the high density of the pathogen in the tissues. Non-selective media are generally used for growth of pure culture of *R. solanacearum especially in* retrieve cultures from frozen stocks or for successive plating of cells.

A. Casamino acid-Peptone-Glucose (CPG) medium (Kelman, 1954)

Casamino acid (casein hydrolysate)	1 g
Peptone	10 g
Glucose	5 g
Agar	17 g

Adjust pH to 6.5-7.0 if necessary. Autoclave at 121°C for 20 minutes.

On solid medium, colonies of *R. solanacearum* usually are visible after 48-72 hours of incubation at 28ºC. Colonies of the normal (or virulent type) are white or cream-coloured, irregularly-round, fluidal, and opaque; and colonies of the mutant (or non-virulent) type are uniformly round, smaller, and butyrous (dry). This shift from virulent to non-virulent bacterial cells occurs during storage or under oxygen stress in liquid media.

B. Triphenyl tetrazolium chloride (TTC or TZC) medium (Kelman, 1954)

Prepare one litre of CPG medium as per details given above.

Adjust pH to 6.5-7.0 if necessary. Autoclave at 121°C for 20 minutes. After autoclaving cool the medium to 55°C and add 5 ml of a 1% stock solution of 2, 3, 5-tripheny tetrazolium chloride. The stock can be filter sterilized or autoclaved for 5 minutes at 121°C, and stored at 4°C or frozen.

2.4.2 Semi-selective Media

Semi-selective media can be used for isolation of the pathogen from non-symptomatic plants or from water and soil samples. Although widely used, they do not support growth of all *R. solanacearum* strains and/or do not suppress growth of all related or unrelated Gram-negative bacteria.

A. SM-1medium (Granada and Sequeira, 1983a)

Prepare 1 litre of TTC (or TZC) medium as per details given above.

Autoclave at 121°C for 20 minutes. After autoclaving cool the medium to 55°C and add 5 ml of a 1% stock solution of 2, 3, 5-tripheny tetrazolium chloride. The stock can be filter sterilized or autoclaved for 5 minutes at 121°C, and stored at 4°C or frozen. One litre of medium add Merthiolate tincture 5-50 µl. Also add Crystal violet 50 mg, Polymyxin β sulfate 100 mg, Tyrothricin 20 mg, Chloromycetin 5 mg, and Cycloheximide 50 mg.

[(Merthiolate tincture * contains 1 part Merthiolate per 1000 parts of 50% alcohol. Determine the best concentration to suppress local microflora, as suggested (Granada and Sequeira, 1981, 1983). Dissolve in 5 ml of 70% ethanol 30 minutes prior to use)].

B. Modified SMSA medium (Elphinstone *et al*., 1996; French *et al*., 1995) Prepare 1 litre of TTC (or TZC) medium, except substitute glycerol (5 ml per litre) for the glucose:

Casamino acid (casein hydrolysate)	1 g
Peptone	10 g
Glycerol	l5 ml
Agar	17 g

Adjust pH to 6.5-7.0 if necessary. Autoclave at 121°C for 20 minutes. After autoclaving cool the medium to 55°C and add 5 ml of a 1% stock solution of 2, 3, 5-tripheny tetrazolium chloride. The stock can be filter sterilized or autoclaved for 5 minutes at 121°C, and stored at 4°C or frozen. One litre of medium add Crystal violet 5 mg, Polymyxin β sulfate 100 mg, Bacitracin 25 mg, Chloromycetin 5 mg, Penicillin 0.5 mg, When inhibition of fungal contaminants is desirable, Cycloheximide 100 mg may be added into the medium. Dissolve in 5 ml of 70% ethanol 30 minutes prior to use. The following chemicals are added in one litre of medium as Crystal violet *5 mg, Polymyxin β sulfate *100 mg, Bacitracin *25 mg, Chloromycetin *5 mg, Penicillin * 0.5 mg, When inhibition of fungal contaminants is desirable, Cycloheximide *100 mg may be added into the medium. * Dissolve in 5 ml of 70% ethanol 30 minutes prior to use.

Immunofluorescence colony staining (IFC), which combines bacterial colony-growth and serological methods, allows sensitive and quantitative detection of several plant pathogenic bacteria in complex environments with high microbial backgrounds like potato peel extracts (Van der Wolf *et al*., 1998). Van der Wolf *et al*. (2000) reported that IFC was a sensitive method for quantitative detection of *R. solanacearum* bv 2 in soil. Elphinstone *et al.* (1996) found consistent positive results with IFC for the detection of *R. solanacearum* in potato tuber extracts only when 2×10^4 to 2.5×10^6 cfu/ ml were present. Imazaki and Nakaho (2010) used pyruvate amended modified SMSA medium to revive the viable but nonculturable (VBNC), which degrades hydrogen peroxide or serve an antitoxidant function in semiselective medium.

Sodium pyruvate @ 5 g/l most improved the sensitivity of *R. solanacearum* detection.

2.5 MORPHOLOGICAL, BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF *R. SOLANACEARUM*

2.5.1 Potassium Hydroxide (KOH) Test

It is a preliminary test of Gram-staining to differentiate Gram-negative and Gram-positive bacteria within a short period. The destruction of the cell wall of Gram-negative bacteria and subsequent liberation of DNA, which is very viscid in water and produces the string of slime. Gram-positive wall is more resistant to KOH and it remains intact, and DNA is not released. 1-2 drops of 3% KOH is kept onto a clean microscopic glass slide. 24 h old culture of *R. solanacearum* from slant with help of a cooled sterile loop is mixed with KOH until an even suspension and lifts the loop from the slide. The *R. solanacearum* being Gram-negative bacteria will become gummy upon mixing with a loop and a string of slime is lifted.

2.5.2 Gram Staining

Gram reaction is essential for primary division of the bacteria. The Gram-staining differentiates Gram-negative and Gram-possitive bacteria on the basis of chemical composition of cell wall of the bacteria. Gram-staining involves the use of primary stain (crystal violet), the trapping reagent (iodine solution), decolouring agent (95% alcohol) and counter stain (safranin). Most plant pathogenic bacteria are the Gram-positive cells stain purple and the Gram-negative cells red.

Procedure:

- On a clean slide, dry a thin spread bacterial film in air, without heat. Then lightly flame the underside of the slide twice to fix the bacteria to the slide.
- Flood the smear with crystal violet solution for 1 min.
- Wash in tap water a few seconds. Drain off excess water, and lightly blot dry on a paper towel.
- Flood the smear with iodine solution for a minute.
- Wash in tap water a few seconds; blot dry.
- Decolourized with solvent, e.g., ethyl alcohol, until the solvent flows colourlessly from the slide (about 30 seconds) blot dry. (If decolourizer is used longer, the Gram-positive bacteria may lose colour).
- Rinse in tap water for about 2 seconds.
- Counter stain for about 10 seconds with safranin solution.
- Wash briefly in tap water. Blot dry and examine.

Ralstonia solancearum is stained red under microscope with 100 × objective lens and oil emulsion.

2.5.3 Arginine Hydrolase Test

This test is important for distinguishing *Pseudomonads*.

Medium

Peptone	1.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Agar	3.0 g
Phenol red	0.01 g
L-arginine	1.0 g
Distilled water	1000 ml
pH	7.2

Dispense the medium in 5 ml quantities in tubes and autoclave. Stab inoculate the medium with 48 hr growth of the bacterium and cover the medium with sterile liquid paraffin to a depth of 1 cm. Incubate the tubes for 7 days at 25 – 30°C and observe daily. A change of the medium to red indicates the arginine – hydrolase activity. *Ralstonia solnacerum* shows negative arginine hydrolase test.

2.5.4 Nitrate Reduction

Medium

Peptone	10.0 g
Beef extracts	5.0 g
KNO_3 (nitrite free)	3.0 g
Distilled water	1000 ml

Dispense the medium about 10 ml into tubes, autoclave and cool. Then inoculate with the strain in question and plug each tube with 3% noble agar. Grow at 27°C up to 5 days is recorded as a positive test for denitrification. Add a few drops of sulphanilic acid (0.8% in 5N acetic acid and dimethyl-alpha-naphthylamine (0.5% in 5N acetic acid) to the nitrate broth culture. Nitrite is present if the mixture becomes distinct pink or red. No colour would mean that nitrate is present as such or has been reduced to ammonia and free nitrogen. To confirm either of these two possibilities add few zinc crystals to the above broth-reagent mixture and shake for a few minutes. Nitrates are present without reduction if the broth becomes pink or red. No colour in either of the above two tests would mean that nitrate is reduced to ammonia or free nitrogen. However, *Ralstonia* species cannot reduce nitrogen.

2.5.5 Oxidase Test (Fahy and Persley, 1983)

The oxidases are enzymes catalyzing the transfer of hydrogen directly to molecular oxygen, resulting in the formation of water.

$$2H_2 + O_2 = 2H_2O$$

For oxidase test, streak a 24-48 h slant growth of the test bacterium on a filter paper saturated with 1 % tetramethyl-para-phenylene-diamino-dihydrochloride. The reaction is positive if a red

or purple colour appears within 10 seconds. The reaction is positive if the colour appears in 10-60 seconds (Kovacs 1956).

2.5.6 Fluorescent Pigments Production (Schaad *et al.,* 2001)

King *et al.*'s B medium (KB)	
Proteose peptone	20.0 g
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g
Glycerol	15 ml
Agar	15.0 g
Distilled water	1000 ml

The KB medium is used for detection of fluoresce in a fluorescent green or blue water soluble, chloroform insoluble Petri dish pigment (*i.e.*, pyoverdine siderophores). After 24-48 h growth at 27°C, colonies are examined for fluorescence with a long wavelength (366 nm) ultraviolet lamp. Impurities such as iron will repress pigment formation and quench the fluorescence of formed pigments. However, *R. solanacearum* did not produce fluorescent pigment on KB medium. Sometimes, it produces brown pigment during storage of the culture.

2.5.7 Hypersensitive Reaction on Tobacco Leaves

Most of the phytopathogenic bacteria produce hypersensitive reaction on tobacco leaves. Prepare a dilute suspension (10^7 cfu/ml) of the test bacterium and inoculate by Klements (1953) injection infiltration method. Record the observation for quick necrosis of the tissue within 24 h.

2.5.8 Detection of Poly-β-hydroxybutyrate Granules

(a) Prepare a smear of bacterial ooze from the infected tissue or from a 48-hour culture on YPGA or SPA medium on a microscope slide.

(b) Prepare positive control smears of a biovar 2 strain of *R. solanacearum* and, if considered useful, a negative control smear of a known PHB negative sp.

(c) Allow to air dry and pass the lower surface of each slide rapidly above a flame to fix the smears.

(d) Stain preparation with either Nile blue or Sudan Black and observe microscopically as described below.

2.5.9 Nile Blue Test

(a) Flood each slide with 1% aqueous solution of Nile blue A and incubate for 10 minutes at 55°C.

(b) Drain off the staining solution. Wash briefly in gently running tap water. Remove excess water with tissue paper.

(c) Flood the smear with 8% aqueous acetic acid and incubate for one minute at ambient temperature.

(d) Wash briefly in gently running tap water. Remove excess water with tissue paper.
(e) Re-moisten with a drop of water and apply a coverslip.
(f) Examine the stained smear with an epifluorescence microscope at 450 nm under oil immersion at a magnification of 600 to 1000 using an oil or water-immersion objective.
(g) Observe for bright orange fluorescence of PHB granules. Also observe under transmitted normal light to ensure that the granules are intracellular and that cell morphology is typical of *R. solanacearum*.

2.5.10 Sudan Black Test

(a) Flood each slide with 0.3% Sudan Black B solution in 70% ethanol and incubate for 10 minutes at ambient temperature.
(b) Drain off the staining solution and wash briefly in tap water, removing excess water with tissue paper.
(c) Dip the slides briefly in xylol and blot dry on tissue paper. *Caution: Xylol is harmful, take necessary safety precautions and work in a fume cupboard*.
(d) Flood the slides with 0.5% (w/v) aqueous safranin and leave for 10 seconds at ambient temperature. *Caution: Safranin is harmful, take necessary safety precautions and work in a fume cupboard*.
(e) Wash in gently running tap water, blot dry on tissue paper and apply a coverslip.
(f) Examine stained smears with a light microscope using transmitted light under oil immersion at a magnification of 1000 using an oil-immersion objective.
(g) Observe for blue-black staining of PHB granules in cells of *R. solanacearum* with pink-stained cell walls.

2.6 DIFFERENTIATION OF *RALSTONIA* SPECIES

Ralstonia contains only five recognized species and among them *R. insidiosa, R. mannitolilytica* and *R. pickettii* have been isolated from human clinical samples and latter two can be pathogenic (Ralston *et al*., 1973; De Bacre *et al*., 2001; Coenye *et al.,* 2003). *R. pickettii* has often been used as an out group in genetic studies of *R. solanacearum*, but R. *mannitolilytica* is more closely related to *R. solanacearum* (De Bacre *et al*., 2001; Vandamme and Coenye, 2004; Vaneechoutte *et al*., 2004). *R. solanacearum*, which causes lethal wilting disease (Table 2.2).

Table 2.2 Biochemical tests to distinguish *Ralstonia solanacearum* from some related non-fluorescent plant pathogenic bacteria, belonging to *Acidovorax, Burkholderia* and *Pseudomonas*

Test	*R.solanacearum*	*Burkholderia cepacia*	*Burkholderi gladioli*	*Burkholderia aryophylli*	*P. corrugate*	*Acidovorax avenae*
Diffusible pigment	+	+	+	+	–	–
Oxidase	–	+	V	+	+	+
Agrinine dihydrolase	–	–	–	+	–	–

Contd...

Contd...

Nitrate reduction	–	–	–	+	+	+
Growth at 41°C	–	V	V	+	–	+
Oxidation of:						
Galactose	+/V	+	+	–		
Glycerol	+/W	–	–	+W		
Mannose	+/V	–	+	–		
Utilization of:						
Cellobiose	V	+	+	V	–	
Trehalose	V	V	+	+		
D-Arabinose	–	+	+	+	V	–
D-Tartrate	+/V	-/W	+	–		
Mannitol	V	+	+	+		+
Sorbitol	V	+	+	+		+
L-Rhamnose	–	–	–	–	–	
Levulinate	V	+	-/W	–		
Sucrose	+	+	+	+		–
Glucose	+	+	+	+	+	+
Benzoate	V	–/V	+	–		
n-Propanol	+	+	–	+W	–	–
β-Alanine	V	–	–	–	–	+
Betaine	–	+	+	+		
L-Arginine	–	+	+	+		
L-lysine	–	+	–	V		
Heptanoate	–	+	–	–		
D-Fucose	–	+	+	+		
D-Raffinose	–	V	–	+		

+ = positive reaction; – = negative reaction; V = variable; W = weak

Table 2.3 Differentiation of two *Ralstonia* species from *Pseudomonas syzygii* and the blood disease bacterium of banana (Eden-Green, 1995)

Test	*R. syzygii*	*B. D. Bacterium*	*R. solanacearum*	*R. picketii*
Colonies on TTC medium	Tenacious, minute	Viscid, 5 mm	Fluidal, > 5 mm	ND
Motility	–	–	V	+
Growth at 37°C	–	+	+	+
Growth at 41°C	–	–	–	+

Contd...

Contd...

NaCl tolerance	< 1%	< 1.5 %	< 2.0 %	ND
Nitrate from nitrite	V	–	+	+
Gas from nitrate	–	–	V	+
Tobacco HR	–	–	+ (Systemic infection with stains from tobacco)	ND
Plant pathogenicity and host association	Clove	Banana	Solanaceae, Musaceae, etc.	Bacterium in humans; intracellular growth in *Acanthamoeba* spp.

ND = not determined; V = variable

2.6.1 Characterization of Biovar of *Ralstonia solanacearum*

In India, race 1 and race 2 and biovar 2, 3 and 4 (Hayward, 1976) are prevalent and race 2 and biovar I have not been recorded. In cool and humid hilly area race 3 and biovar 2 are prevalent whereas on the plain and plateau area of West Bengal race 1 and biovar 3 are prevalent. Biovar 4 is prevalent only at a few locations (Shekhawat, 1976; Sinha, 1985). Primarily the biovar 3, 4 and 5 are Asian type whereas biovar 1 and 2 are American type (Cook *et al*., 1989). Recently, the race 1 has been reported from Andaman (Ansari, 1990) and three races have been reported infecting different crops from various locations of Assam by Nath *et al*. (1996). Prior *et al*. (1990) has studied the aggressiveness of pathogen on tomato. The strain of *R. solanacearum* can be differentiated into 5 biovars (Hayward, 1964; He *et al*. 1983) according to their ability to oxidize several disaccharides (lactose, maltose and cellobiose) and hexose alcohols (mannitol, sorbitol and dulcitol). Singh, *et al.* (2010) surveyed in northern and eastern states of India such as Jammu & Kashmir, Himachal Pradesh, Uttarakhand, Jharkhand and West Bengal and isolated 68 isolates, out of which 58 isolates were categorized as biovar 3 and remaing 7 biovar 4. The biovar 3 and 4, causing wilt in solanacearous crops belong to race 1.

Symptom identification (Fig. 2.1) is the first step for early diagnosis of bacterial wilt of tomato. Accurate identification of *R. solanacearum* from either symptomatic or asymptomatic plants and from water or soil samples demands multiple microbiological and molecular methods. A battery of complementary tests that differ in their sensitivity and/or specificity should be used for field or laboratory analyses for unambiguous identification of bacteria to species and biovar. Screening tests can facilitate early detection and identification of bacteria in potentially infected plants or contaminated soil and water samples by *R. solanacearum*. They cannot be used to identify the race or biovar of the organism. For differentiation of biovars of *Ralstonia solanacearum* based on various tests including utilization of single alcohols and carbohydrates such as dextrose, mannitol sorbitol, dulcitol trehalose and oxidation of lactose, maltose, D(+) cellobiose and nitrite from nitrate and gas from nitrate are practiced.

Basal medium

$NH_4H_2PO_4$	1.0 g
KCL	0.2 g
$MgSO_4.7H_2O$	0.2 g
Peptone	1.0 g

Agar	3.0 g
Bromothymol Blue	80 mg
Distilled water	1000 ml
pH	7.0-7.1

Autoclave the medium at 121°C for 20-30 min and cool it to 50 to 60°C. Prepare 10% aqueous solution of different carbon sources such as dextrose, mannitol sorbitol, dulcitol trehalose and oxidation of lactose, maltose, D(+) cellobiose. Sterilize all the carbon sources with 0.22 µm membrane filter except dulcital which is sterilized by autoclave separately. Add 1% of the carbon sources into the basal medium. After thorough mixing, dispense 3 ml of the molten medium into the sterilized culture tubes and allow to solidify. Add 100 µl of 48 h old culture of *R. solanacearum* into the basal medium and incubate at 28-30°C and observe the tubes on 2, 4, 7 and 10 days. The change in colour of medium from an olivaceous green to yellow (acid pH, <6) indicates oxidation of the carbohydrate. Those biovars capable of oxidizing the disaccharides will take a few days more to give a clear positive result. The *R. solanacearum* has been divided into five biovars based on utilization and oxidation of carbon sources (Table 2.4 & Table 2.5). Hayward (1964) has classified the bacterium on the basis of bio-types (now biovars) or biochemical types. He used number 1, 2, 3, 4 and 5 biotypes for this subdivision, which appear to have little relation of the strain as pathogen. Biovar 2 further subdivided by using additional tests utilization of carbon sources and pectolytic activity. Bacterial wilt of potato in temperate and subtropical regions and at high altitudes in the tropics worldwide is caused by biovar 2 (race 3) strains with the phenotype D-ribose negative, trehalose negative and *meso*-inositol positive. This phenotype is RFLP group 26 in the classification of Cook *et al*. (1989). A dinstict phenotype of biovar 2 occurring in parts of Chile and Colombia, South America is D-ribose negative, trehalose positive and *meso*-inositol negative (Hayward, 1995; Hayward *et al*., 1990). In RFLP group 27, most of these isolates do not produce nitrite from nitrate, a property of universal among all other biovar 2 strains. A third phenotype of biovar 2, which occurs mainly in Peru and Brazil, is D-ribose positive, trehalose positive and meso-inositol positive (Table 2.5). This type of biovar is also referred as N2 (Cook and Sequeira, 1994). Or biovar 2T to reflect its low land tropical origin (French *et al*., 1995) corresponds with RFLP groups 29-31, 33, 36 and 39 (Cook and Sequeira, 1994). Prevalence of biovar 3 strains in different agro-climatic zones of India has been reported (James *et al*., 2003; Singh *et al*., 2010). High incidence of biovar 3 strains also exhibit that these have broad host range and are well adapted to various degrees of biophysical conditions and other biological factors such as interactions with various microfloras in soil. Biovar 2 (race 3) strains are widely distributed in Asia including India, Pakistan, Bangladesh, China and Philippines and Middle East countries (Lebonan and Iran) (CABI/EPPO, 1998). However, biovar 2 strains have more adaptation to cooler temperature. These strains have limited host range, affecting only potato as compared to biovar 3 strains (Lemessa and Zeller, 2007). Danial *et al*. (2006) reported that *R. solanacearum* strains race 3 biovar 2A were mainly responsible for outbreak of potato brown rot in Europe. Chakraborty *et al*. (1994) reported the occurrence of *P. seudomonas solanacearum* biovar 3 on eggplant, tomato and potato in West Bengal. Li, *et al*. (2006) have made genetic analysis of resistance to bacterial wilt and identification of an associated AFLP marker in eggplant.

Contd...

3	Ceylon (Sri Lanka)	Seneviratne and De, 1969
	USA	Hayward, 1976
	Taiwan	Shaw, 1984; Hsu, 1991
	South-East Queensland	Peterson *et al.*, 1983
	Assam	Baruah and Deka, 1995
	West Bengal	Chattopadhaya *et al.*, 1994
	Bihar	Sinha, 1985
	Brazil	Quezado-soares and Lopes, 1994
	Nepal	Bhattrai *et al.*, 1998
	Chegalu (China)	Ling *et al.*, 1983; Shuai Zhengbin *et al.*, 1997
	Singapore	Yik *et al.*, 1994
	Kerala	Mathew and Peter, 2004
	Ranchi	Singh *et al.*, 2010
	Himachal Pradesh	Singh *et al.*, 2010
	Uttarakhand	Singh *et al.*, 2010
	Jammu and Kashmir	Singh *et al.*, 2010
4	Plains and plateau area of	Shekhawat, 1976; Sekahwat *et al.*, 1978
	West Bengal and Bihar	Sinha, 1985
	Japan	Kayamura *et al.*, 1985
	Papua New Guinea	Shaw, 1984
	Singapore	Yik *et al.*, 1994
	Saudi Arabia	Abadalla *et al.*, 1999
5	Singapore, Ranchi (Jharkhand)	Shuai Zhengbin *et al.*, 1997; Sharma and Kumar, 2005

Yadessa *et al.* (2010) reported that *R. solanacearum* race 3 biovar 2 (phylotype II) is the causal agent of bacterial wilt of tomato, the most destructive bacterial disease of this crop in Ethiopia.

2.6.2 Identification of races of *R. solanacearum*

There are differences in cultural and physiological properties among the isolates of *R. solanacearum* (Table 2.7). Race designation was determined by inoculating seedlings of 'Hick' Tobacco (*Nicotina tabacum*), '*Bonny Best'* tomato, 'Golden Summer' pepper (*Capsicum annuum*), and 'Mini Finger', eggplant (*Solanum melogena*) (Buddenhegen *et al*., 1962). Five strains from the Quincy production area and sixth strains (K60) where isolated from tomato in North Carolina by Kelman and Person (1961). Three races of bacterium *R. solanacearum* have been recognized on the basis of host range studied (Buddenhagen and Kelman, 1964; Morton *et al.,* 1965). They reported that race 1 affecting solanaceae and other plants, race 2 from isolates affecting bananas and *Heliconias* and race 3 for isolates primarily affecting potato whereas Kishore *et al.* (1991) reported new record for race 1 in potato in high hills from India. Lozano and Sequeira (1970) reported that the race of *P. solanacearum* can be differentiated by a leaf infiltration technique. Duke *et al.* (1965) have identified race 3 from indigenous strain which were highly virulent to tomato, but only slightly virulent to flue cured tobacco in Georgia

whereas 3 biotypes were reported from Ceylon by Seneviratne and De (1969) who reported that biological environmental factors play a part in differentiating the distribution of biotype. The global distribution of biovars of race 1 of tomato is shown in Table 2.6.

Table 2.7 Race determination in *R. solanacearum**

Host reaction	*Race*		
	1	*2*	*3*
Tomato/aubergine	Wilting	No reaction	Wilting
Tobacco cv. White Burley plants (stem inoculation)	Wilting	No reaction	No reaction
Tobacco cv. White Burley leaves (hypersensitivity test)	Necrosis (48 h) and wilting (7-8 days)	HR (12 – 24 h)	Chlorosis (2-8 days)
Musa accuminata	No reaction	Wilting	No reaction

*Race 4, pathogenic to ginger and a few other hosts and race 5, pathogenic to mulberry only, not included here (Janse, 1991)
Source: OEPP/ EPPO Bulletin 34: 173- 178.

2.6.3 Serological Characteristics of *R. solanacearum*

It is interesting to know whether there is a serological relationship between *R. solanacearum* and the other phytopathogenic or saprophytic bacteria which will be useful to know whether this species could be serologically related to the other phytopathologenic bacteria affecting the same host-plant. The technique used for serological studies on *R. solanacearum* is very similar to those used generally in the serology of other phytopathologenic bacteria.

Preparation and injection procedure of antigenic material: The antigenic material is usually prepared from whole bacterial cells. The whole cell may be living (Digat, 1968) or heat-killed at 100°C for 2 hours (Kojima and Buddenhegen, 1969). Sonicated cells were used once (Mortan *et al*., 1965). These antigens are injected intravenously, but doses and frequency of injections were variable among the various authors.

Antiserum testing: The antisera can be tested by several techniques. The classical agglutination technique is the most frequently used. However, some different techniques, *viz*., ring test (Perez, 1962), hemagglutination (Perez, 1967), and complement-fixing test have also been used.

2.6.4 Problem of Serological Specificity for Races and Strains

Serum specificity is sometimes questionable. The often-heard criticism of serology is that frequently the serological characters cannot be related to pathogenicity characters. However, a relationship could be shown between these two characters if the precise nature of pathogenicity characters was known. In other words, if it would be possible to extract and to purify protein molecules related to or controlling pathogenicity, it would be possible to use them as antigens. Antisera for the pathogenicity molecules could be obtained and a precise relationship between serology and pathogenicity could be finally established. In other words to become concrete, this hypothesis needs a systematic analysis of antigenic entities of the bacterium. Somatic antigen and the extracellular glycoprotein antigen are among these.

of the colonies. Otherwise, agglutination tests can be used directly with exudates from several infected plants. In this way, the bacterium can be directly detected in potato, tomato, eggplant, sweet pepper and banana. The most commonly used assays for bacteria detection and identification are agglutination, enzyme-linked immunosorbent assay (ELISA), immunofluorescence (Van der Wolf *et al*., 2000), lateral flow strip tests or flow-through assays immunodiagnostic assays using *R. solanacerum* specific (Danks and Barker, 2000; Van der Wolf *et al*., 2000). Antibodies (also known as immunoglobulins) are proteins that are used by the immune system to identify and neutralize foreign objects, such as bacteria. Due to their specificity, they are commonly used in biology for detection and identification of microorganisms.

A more suitable subtle detection technique is immunofluorescence, which provides a means for detecting the bacterium in the plant, water and soil (Berniac and Coleno, 1974). In this case, practical methods for a bacterial analysis of soil have to be improved since many serological cross-reactions with saprophytic soil bacteria could occur. The IF technique can be also used for detecting the bacterium in the host-plant and for studying the rate of spread of the pathogen.

2. **Taxonomy and Epidemiology:** The study of Digat and Cambra (1976) revealed that race and strain-specific agglutinating antisera could be obtained by absorbing them and using a convenient routine-dilution. Immunodiffusion technique is more practical for differentiating the subtle serological relationships among the strains.
3. **Disease resistance and host-pathogen relationship:** Bacterial cell immunolabelling provides a means of following the spread of bacterium from the roots to the top of the host-pathogen. Frequently, bacterial wilt resistant hybrids (tomato, potato) may be symptomless carrier of *R. solanacearum*. The very sensitive immunofluroescence technique provides a means for detecting small numbers of bacteria in the tolerant host-plant. The immunolabelling technique such as IF, immunoenzyme and immunoferritin techniques can contribute in clarifying the unknown cellular relationship, in particular by observing tissue sections.

Serology techniques are relatively low-cost and easily performed tests for routine *in situ* use. Immunoassays are being applied routinely for the detection of plant pathogens in plant material and soil and tests such as ELISA have demonstrated the sensitivity and specificity required to replace time-consuming and expansive assays like indicator-plant inoculation and dilution plating. Serological techniques also have the advantage that immunoassays are well established techniques for detection and identification of bacterial species.

Nucleic acid probes and PCR amplification offer highly specific and sensitive means to detect pathogens and their strains. Nucleic acid probes have the advantage over serological methods in that a test can be made that targets any specific surface components. Antigens present at one stage of development may differ in different media. Conversely, the genome of an organism is unlikely to change and give a false negative result depending on the developmental stage, growth conditions or extraction method employed. It is also unlikely that antibodies, even monoclonal, can be used in the same way as DNA probes to distinguish between very closely related strains. Although species-specific antibodies for some bacteria have been developed, relatively small genetic differences between isolates of the outside species would not usually be manifested as changes on the outside of the bacterial cell. DNA probes often provide the

only reliable method of detecting these differences between closely related strains. However, for a rapid and reasonably sensitive assays of total bacterial numbers of a particular species, immunoassays may be the method of choice.

2.6.7 Immunological Assays

Protocol for soil assay:

1. Weigh 1 g of soil and suspend in 2 ml of buffer A.
2. Shake vigorecesly for 1 minute.
3. Allow settling for 5 min. and remove supernatant.
4. Coat the wall of immunoplate with this supernatant.
5. Incubate in a moist chamber at 37°C for one hour, or overnight at 4°C.

Protocol for plant assay:

1. Cut a 1cm section from the stem base (at or close to the intersection between stem and tap root), and weigh.
2. Macerate mechanically and resuspend in buffer B at the rate of 1g/ml.
3. Allow settling for 30 min. and remove supernatant.
4. Incubate in a moist chamber at 37°C for one hour, or overnight at 4°C.
5. Wash three times in buffer D by submerging the plate in the buffer and then flicking it out to empty it. Leave for 5 min., flick-out and then bang dry on sheet of tissue.
6. Dilute *R. solanacearum* antibodies 1:2000 in buffer add 100 µl to each wall.
7. Incubate and wash as in step 5 & 6.
8. Dilute anti-rabbit peroxidase conjugate 1:5000 in buffer C and add 100 µl to each cell wall.
9. Incubate and wash as in step 5 & 6.
10. Dissolve one TMB tablet in 10 ml of buffer and add 100 µl per well.
11. Leave at room temperature untill a colour change is obtained observed (5-20 min).
12. Stop the reaction by adding 25 µl to 3 M sulphuric acid to each wall.
13. Read the absorbance by 450 nm, or record the colour change visually .

Materials required:

(i) Immunno plates (ELISA Plates)
(ii) Polyclonal antibody of *R. solanacearum*
(iii) Anti-rabbit peroxides conjugate
(iv) 3,3,5,5 tetra methyl benzedrine (TMB) tablets

Stock preparation for immunological assay:

(i) 3M sulphuric acid
(ii) 10% Tween-20

to produce wilt. The colonization index at mid stem was more useful in corn resistant under favourable condition. When environmental conditions were unfavourable to wilt, colonization index at collar level deserted resistant genotype clearly. This result framed the basis for a model for predicting the degree and stability of resistance (Prior *et al*., 1996). Rajeshwari *et al*. (1998) have developed a technique for detection of *R. solanacearum* in tomato. This serological assays so far the detection of pathogen were able to provide information as to the presence or absence of the pathogen in soil and plant, however, they could not discriminate between virulent and avirulent strains of the pathogen and were not specific to strain and races. In this technique, virulent bacterial cells (encapsulated with mucin) from tomato seeds were used as antigen and polyclonal antisera were developed in rabbit using a classified immunization protocol. Antisera thus developed were examined for the antibodies titre, sensitivity, specificity, rapidity and the efficacy of the antibody for identifying the potential for the application of this assay to the screening of the infected plant materials and seeds. The results indicate that anti-tomato *R. solonacearum* (i) has a good antibody titre of 1:10000; (ii) can detect a few as 100 bacterial cells/ml; (iii) *R. solonacearum* is tomato specific (it reacted with tomato *R. solonacearum* and not isolates from chilli (capsicum) or brinjal; (iv) is reactive to all isolates of *R. solonacearum* from tomato; (v) is not cross-reactive with non-pseudomonades (vi) is virulent strain specific as it recognizes the virulent exopolysaccharides component an anti-determinants and (vii) reactivity could be correlated well with the degree of infection in tomato seeds and plant materials. Thus ELISA devloped is sensitive, specific and rapid, therefore, suitable for detection of *R. solanacearum* isolates from tomato seeds during routine assays. Mc Garvey *et al*. (1999) have used this technique and reported that bacterial population in susceptible cultivars produced greater amounts of EPS 1 per plant than in resistant cultivars. Opina and Miller (2005) evaluated ELISA and immunostrip to detect *R. solanacearum* from 505 eggplant samples from 48 fields in the Philippines. They reported that both the ELISA and immunoStrip were the same for 91% of the samples, while remaining 9%, immunostrip results were positive, whereas ELISA were negative. Isolates of *R. solanacearum* belonging to biovar 1 and biovar 2A are successfully detected by double antibody sandwich- enzyme linked immunosorbent assay (DAS- ELISA) at low population levels after incubation of soil suspensions for 48 h at 30°C in a semi selective broth containing a potato tuber infusion. Detection thresholds of 20 and 200 cfu/ g of inoculated soil were obtained for biovar 1 and biovar 2A respectively. In naturally infested soils, average sensitivities of 97.6 and 100.9 cfu/g cells of soil were obtained for biovars 1 and 2A respectively (Priou *et al*., 2006). *R. solanacearum* was also detected from tomato irrigation ponds and aquatic weeds such as *Polygonum pensylvanicum* and *Hydrocotyle ranunculoides* in or surrounding the irrigation ponds to determine their role as alternative hosts or the source of the pathogen by using enrichment medium SMSA and ELISA.

Enrichment-ELISA Protocols

In this method, combining ELISA, which uses specific monoclonal antibodies, with prior enrichment of stem greatly, improves the sensitivity of detection of bacterial pathogens (Gorris *et al.,* 1994; Lopez *et al.,* 2001). It is effective because of low sensitivity of ELISA for bacterial detection using specific monoclonal antibodies (approximately 10^5-10^6 cfu/ml) and need to improve this sensitivity to detect latent infections of quarantine bacteria (Janse, 1988,

2006; Gorris *et al.,* 1994; Louws *et al.*, 1999). The use of optimized enrichment for each plant pathogenic bacterium allows its specific multiplication in the sample before detection. Specific protocol is required for *R. solanacearum*. The medium temperature, duration and incubation conditions of the enrichment are crucial for optimizing it and improve the detection sensitivity (Gorris *et al*., 1994; Lopez *et al*., 1997). To improve the detection of *R. solanacearum* biovar 2 from water samples at low temperature, enrichment for 72 h in modified Wilbrink liquid medium was set up that can be combined with DASI-ELISA using monoclonal antibodies. It was able to detect viable but nonculturable *R. solanacearum* cells (1-10 cfu/ml) (Caruso *et al.*, 2005). *R. solanacearum* biovar 2A was detected from stems of symptomsless plants before harvest of the potato crops by using double antibody sandwich (DAS)-ELISA and indirect ELISA on nitrocellulose membrane after enrichment of the plant extracts in a semi-specific broth (Priou *et al.,* 2010). A simple method is required for rapid detection of plant pathogenic bacteria for testing large number of samples by non-experienced technicians. For this purpose, tissue print ELISA and lateral flow devices have been designed for bacteria. Although detection specificity is very high when using the appropriate monoclonal antibodies. The sensitivity is low for detecting bacteria and they are more appropriate for analyzing plants with symptoms. The lateral flow devices kits are based on the existing technology similar to a pregnancy test kit and such kits are only available for a few bacteria. A lateral flow device kit developed by Central Science Laboratory, U. K., permits detection of *R. solanacearum* in 3 min, in single step (Danks and Baker, 2000). Rapid ImmunoStrip® is available from Agdia Inc.

2.6.9 Flow Cytometry

Immunodiagnostic detection has been enhanced by the development of flow cytometry. It can be used for identification and quantification of cells or other particles as they pass individually through a sensor in a liquid stream. Cells are identified by fluorescent dyes conjugated to specific antibodies and multiple cellular parameters are determined simultaneously based on the cells, fluorescence and its ability to scatter light. The cells may be sorted electronically, permitting purification and/or culture of subpopulations of selected cells for further confirmatory tests (Alvarez and Adams, 1999; Alvarez, 2001). Sample is prepared as cell suspensions are filtered to remove large particles then stained with fluorochrome labelled antibodies. Fluorescent markers for viability (vital stains, such as propidium and hexidium iodide for red fluorescent staining of dead cells and corboxy fluorescein diacetate and calcein AM for green fluorescent staining of viable cells) can be used to differentiate live from dead cells (Van der Wolf and Schoen, 2004). This technique has been applied for detection of *R. solanacearum* and to determine viability of bacteria in seed potatoes (Van der Wolf *et al*., 2004).

Lateral Flow Device

The principles used for rapid lateral flow devices are primarily those of ELISA, but various types of filters are used as the solid support for the initial binding reaction (Danks and Baker, 2000; Elphinstone, 2005). A lateral flow device test kit developed by Central Science Laboratory, U. K., permits detection of *R. solanacearum* in 3 min in a single step (Danks and Baker, 2000).

Contd...

2. Supernatant is filtered, neutralized at pH7 and Precipitated with equal Volume of saturated Ammonium sulphate solution at +4°C.

   ↓

   Sediment eliminate

3. Precipitate is sediment overnight at +4°C and centrifuged at 20,000 × G for 15 minutes

   ↓

4. Pellet is dissolved in original volume of sterile water and dialyzed for 4 days against distilled water. Cruide glycoprotein fraction is named as "Glycoprotein antigen".

Infectivity titration: This method is used for comparison of virulence of *R. solanacearum* strain. Infectivity titration can be used in two ways: to define resistance in host pathogen: and to quantify the virulence of plant pathogen (Lum and Kelman, 1981; Bora and Addy, 1982; Ercolani, 1984). There is an extensive litreature on application of this technique to bacterial disease of plants. Anyone contemplating work in this area should first read the authoritative review of Ercolani (1984) who states that infectivitivity titration is an easy and efficient procedure for measuring virulence of the pathogen and susceptibility of the host in bacterial infection of plants. Both host and pathogen effects can be of either a quantal or a quantitative type. Quantal effects occur as an all-or none response, which by definition can only take either of two mutually incompatible forms, such as alive or dead, healthy or diseased, turbid or wilted, whereas quantitative effects occur as response that can take any of a series of values, e.g., number of local lesions and time to response. Infectivity titration with quantitative response has greater potential for achieving practical results than titration with quantal responses.

Procedure

(i) Prepare serial tenfold dilutions in phosphate buffer so that final concentration of bacteria in colony forming units (cfu's) per ml is ca.10^6, 10^5, 10^4 and $10^{3.}$

(ii) For each of the dilutions inoculate 5 plants. Twenty microlitres aliquots are inoculated by stabbing gently into the axil of the third leaf from the apex of the tomato plants, using micropipette tips, which are left, in place to allow uptake of inoculums. Thus the plants received doses of 20000, 2000, 200 and 20 viable cells, respectively

(iii) A control series of plants is inoculated with 20 microlitres of sterile phosphate buffer (0.05 M, pH 7.0).

(iv) Examine the plants daily for 210 days for the appearance of wilt symptoms using the disease rating scale following Winstead and Kelman (1952).

(v) The infectivity titration of 3 strains of *R. solanacearum*, race 3, biovar D (R. Sol-1 $^{r+c+e+}$, R. Sol-2 $^{r+c+e+}$ and R. Sol-3 $^{r+c+e+}$, rifamycin, chloramphenicol and streptomycin resistant) was studied using tomato plants evaluated 9 and 14 days after inoculation. Two strain R. Sol-1 $^{r+c+e+}$, R. Sol-3 $^{r+c+e+}$, showed lower virulence than wild type(R. Sol-O $^{r-c-e-}$) which did not differ from *R. solanacearum* 2 $^{r+c+e+}$. The evaluation at 14 days showed larger differences between the averages. The infectivity titration can defect strain with similar virulence to the wild type and those which lose virulence during screening to antibiotic resistance (Yosses *et al*., 1998).

2.6.10 Gas Chromatography

Toxin is detected by this method (Baruah and Daka, 1995). This method is for identification of bacteria and statistical analyses of FAME. Profile data were conducted using the MIDI Microbio Identification system, Network, DEL, VERSION 3.6 (Sasser, 1990).

2.7 MOLECULAR CHARACTERISTICS

2.7.1 DNA Marker

DNA genetic markers, such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RFLPs), promise to increase understanding of bacterial wilt resistance dramatically. DNA markers make it possible to monitor inheritance throughout an entire plant genome at high resolution and dissect the contributions of specific genetic loci to complex characters. Aarons *et al*. (1993) have used DNA genetic marker mapping of genes for bacterial wilt resistance in tomato.

Another important application of DNA markers is in breeding for complex, polygenic characters. Often, it is difficult to retain all the genes involved in a complex resistance character throughout the course of a breeding programme. Retaining resistance loci can be specifically challenging if creating an artificial epidemic is difficult or if the disease is associated with a specific environmental condition or plant developmental stage. However, DNA marker tightly linked to the underlying resistance loci can be used as a basis for selection, thereby ensuring that the genes are retained during the course of the breeding programme. The possibility of antigenic contamination (especially by cell wall material or bacterial debris) must be kept in mind.

Harai *et al.* (1998) has used DNA marker for bacterial wilt to evaluate resistance in tomato breeding. However, Bartolla *et al*. (1999) used plasmid DNA and pathogen clone (*R. solanacearum*) GM 11000 for expressing the marker gene was actively multiplied inside the plant vessels. Moreover, experiment in which *R. solanacearum* strain harboring different marker gene were co-inoculated into plant demonstrated that infecting bacterial strain exchange genetic information in the plant by a mechanism of transformation. These assays are based on the use of antibiotics in various test formats to detect and identify any molecules or cells (including bacteria). A biochemical growth test is used for biovar determination of *R. solanacearum*. This test is based on the differential ability of strains of the pathogen to differentially produce acid from several carbohydrate sources, including disaccharides and sugar alcohols. At the sub-species level, identification of strains of *R. solanacearum* can be assessed with several nucleic-acid based methods such as DNA probe hybridization uses the ability of two complementary single-stranded nucleic acids to combine into a single molecule.

2.7.2 DNA Probe Hybridization

Nucleotide probe of known sequence is used to bind complementary strand of undetermined organism for identification. The polymerase chain reaction is a technique that consists of amplifying a DNA molecule exponentially.

1. PCR Protocol (Seal *et al.* 1993)

Oligonucleotide primers

Forward primer OLI-1 5′-GGG GGT AGC TTG CTA CCT GCC-3′

Reverse primer Y-2 5′-CCC ACT GCT GCC TCC CGT AGG AGT-3′

Expected amplicon size from *R. solanacearum* template DNA = 288 bp

PCR Reaction Mix: The following regents are used for study.

Reagent	*Quantity per reaction*	*Final concentration*
Sterile UPW	17,65 µl	
10X PCR buffer (15 mM MgCl2)	2,5 µl	1X (1,5 mM $MgCl_2$)
dNTP mix (20 mM)	0,25 µl	0.2 mM
Primer OLI-1 (20 µM)	1,25 µl	1 µM
Primer Y-2 (20 µM)	1,25 µl	1 µM
Taq polymerase (5U/µl)	0,1 µl	0,5 U
Sample volume	2,0 µl	–
Total volume	25 µl	–

PCR reaction conditions

Run the following programme:

Step I: 96°C for 2 min (denaturation of template DNA) } 1 cycle

II: 94°C 20 s (denaturation of template DNA))

III: 68°C for 20 s (annealing of primers)) 35 cycles

IV: 72°C for 30 s (extension of copy)

V: 72°C for 10 min (final extension) } 1 cycle

VI: hold at 4°C forever

This programme of the duration steps of cycles (II), (III) and (IV) may be changed as per thermal cycler used for PCR amplification.

Restriction Enzyme Analysis of Amplicon

PCR products amplified from *R. solanacearum* DNA produce a distinctive restriction fragment length polymorphism with enzyme *Ava* II after incubation at 37°C.

2.7.3.2 PCR Protocol (Pastrik and Maiss, 2000)

Oligonucleotide Primers

Forward primer Ps-1 5′- AGT CGA ACG GCA GCG GGG G -3′

Reverse primer Ps-2 5′- GGG GAT TTC ACA TCG GTC TTG CA -3′

Expected amplicon size from *R. solanacearum* template DNA = 553 bp.

PCR reaction mix: The following regents are used for study.

Reagent	*Quantity per reaction*	*Final concentration*
Sterile UPW	16,025 µl	
10X PCR buffer (15 mM $MgCl_2$)	2.5 µl	1X (1,5 mM $MgCl_2$)
BSA (fraction V) (10 %)	0.25 µl	0.1 %
dNTP mix (20 mM)	0.125 µl	0.1 mM
Primer Ps-1 (10 µM)	0.5 µl	0.2 µM
Primer Ps-2 (10 µM)	0.5 µl	0.2 µM
Taq polymerase (5U/µl)	0.1 µl	0.5 U
Sample volume	5.0 µl	
Total volume	25.0 µl	

N.B. Originally optimised for MJ Research PTC 200 thermocycler with Gibco Taq Polymerase. Perkin Elmer AmpliTaq and buffer can also be used at the same concentrations.

PCR reaction conditions

Run the following programme:

Step I: 95°C for 5 min (denaturation of template DNA) } 1 cycle
II: 95°C for 30 s (denaturation of template DNA) }
III: 68°C for 30 s (annealing of primers) } 35 cycles
IV: 72°C for 45 s (extension of copy)
V: 72°C for 5 min (final extension) } 1 cycle
VI: hold at 4°C forever

This programme of the duration steps of cycles (II), (III) and (IV) may be changed as per thermal cycler used for PCR amplification.

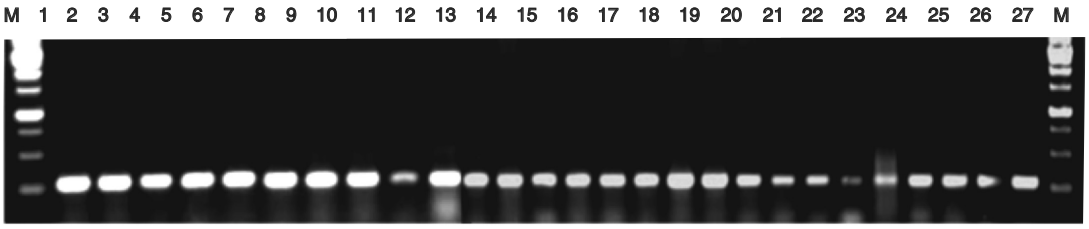

Fig. 2.3 Amplification of fragments of the 16s rRNA gene from *R. solanacearum* isolates separated on an agarose gel showing the 288 bp. Lane 1 & 2: isolates from J& K, Lane 3 - 8: isolates from H. P., Lane 9-19 from Uttarakhand, Lane 20 – 24 from Jharkhand and Lane 25-27 from West Bengal (Singh *et al.,* 2010)

Pastrik and Maiss (2000) have detected *R. solanacearum* in potato tubers by polymerase chain reaction

This programme of the duration steps of cycles (II), (III) and (IV) may be changed as per thermal cycler used for PCR amplification.

Restriction Enzyme Analysis of Amplicons

PCR products amplified from *R. solanacearum* DNA produce a distinctive restriction fragment length polymorphism with enzyme *Bsm* I or an Isoschizomere (e.g., Mva 1269 I) after incubation at 65°C for 30 minutes.

2.7.3.4 Ralstonia solanacearum Biovar-specific PCR Protocol (Pastrik *et al.* 2002)

Oligonucleotide Primers

Forward primer Rs-1-F 5′- ACT AAC GAA GCA GAG ATG CAT TA -3′

Reverse primer Rs-1-R 5′- CCC AGT CAC GGC AGA GAC T -3′

Reverse primer Rs-3-R 5′- TTC ACG GCA AGA TCG CTC -3′

Expected amplicon size from *R. solanacearum* template DNA:

with Rs-1-F/Rs-1-R = 718 bp

with Rs-1-F/Rs-3-R = 716 bp.

PCR Reaction Mixture

(a) Biovar 1/2-specific PCR: The following regents are used for study.

Reagent	*Quantity per reaction*	*Final concentration*
Sterile UPW	12.925 µl	–
10X PCR buffer (15 mM $MgCl_2$)*	2.5 µl	1X (1,5 mM $MgCl_2$)
BSA (fraction V) (10 %)	0.25 µl	0.1 %
dNTP mix (20 mM)	0.125 µl	0.1 mM
Primer Rs-1-F (10 µM)	2.0 µl	0.8 µM
Primer Rs-1-R (10 µM)	2.0 µl	0.8 µM
Taq polymerase (5U/µl)	0.2 µl	1.0 U
Sample volume	5.0 µl	–
Total volume	25.0 µl	–

(b) Biovar 3/4/5-specific PCR: The following regents are used for study.

Reagent	*Quantity per reaction*	*Final concentration*
Sterile UPW	14.925 µl	–
10X PCR buffer (15 mM $MgCl_2$)*	2.5 µl	1X (1.5 mM $MgCl_2$)
BSA (fraction V) (10 %)	0.25 µl	0.1 %
dNTP mix (20 mM)	0.125 µl	0.1 mM
Primer Rs-1-F (10 µM)	1.0 µl	0.4 µM
Primer Rs-3-R (10 µM)	1.0 µl	0.4 µM

Contd...

Table 2.10 List of primer sets for identification of *R. solanacearum* through PCR.

Pathogen	*Probe*		*References*
R. solanacearum	Y2: 5'- CCC ACT GCT GCC TCC CGT AGG AGT-3' OLII: 5'- GGG GGT AGC TTG CTA CCT GCC-3'	288bp	Seal *et al.,* 1993
R. *solanacearum* and related spp.	759: 5'- GTC GCC GTC AAC TCA CTT TCC-3' 760: 5'- GTC GCC GTC AGC AAT GCG GAA TCG-3'	281bp	Ito *et al*., 1998 Opino *et al.,* 1997
R. solanacearum	PS96-H: 5'- TCA CCG AAG CCG AAT CCG CGT CCA TCA C-3' PS96-I: AAG GTG TCG TCC AGC TCG AAC CCG CC-3'	148bp	Hartung *et al., 1998* Seal *et al.,* 1992
R. solanacearum	pehA#3: 5'- CAG CAG AAC CCG CGC CTG ATC CAG-3' pehA#6: 5'- ATC GGA CTT GAT GCG CAG GCC GTT-3'	504bp	Gillings *et al.,* 1993
R. solanacearum race 1 and 3 biovar 1, 2, 3 and 4	RALSF:5'-GCTCAAGGCATTCGTGTGGC-3' RALSR:5'-GTTCATAGATCCAGGCCATC-3'	932bp	Kang *et al.,* 2007
R. solanacearum race 1 biovar 3 & 4	Hrp_rs2F:5'-AGAGGTCGACGCGTACAGT-3' Hrp_rs2R:5'-CATGAGCAAGGACGTCA-3'	323bp	Singh *et al*., 2014b

2.7.3.5 Fluorescence in situ Hybridization (FISH)

Fluorescence *in situ* hybridization is dependent on the hybridization of DNA probes to species-specific regions of bacterial ribosome. It can detect single cells but in practice, the detection level is near 10^3 cells/ml of plant extract and even though has been employed in some recent works (Ercolini *et al.,* 2006). There is a high affinity and selectivity of DNA probes because FISH takes place under very stringent hybridization conditions, where a difference of one nucleotide in a 15-20 oligonucleotide probe targeted to 23S rRNA is sufficient for discrimination. *R. solanacearum* race 3 biovar 2 was detected from potato peels by using this method (Wulling *et al*., 1998; Van der Wolf and Schoen, 2004) and it has been included in official diagnostic protocols in the EU (Directive 2006/63/CE for *R. solanacearum*) and recommended in the EPPO protocol for the same pathogen (EPPO, 2004). The procedure for detecting bacteria from samples is given below.

2.7.3.6 Validated Reagents for FISH Test

Oligo-probes

R. solanacearum-specific probe OLI-1-CY3: 5′-GGC AGG TAG CAA GCT ACC CCC-3′

Non-specific eubacterial probe EUB-338-FITC: 5′-GCT GCC TCC CGT AGG AGT-3′

Fixative solution

(i) Heat 9 ml molecular grade water (e.g., ultra pure water (UPW)) to about 60°C and add 0.4 g paraformaldehyde. Paraformaledhyde dissolves after adding 5 drops of 1N NaOH and stirring with a magnetic stirrer.

Table 2.11 Detection of *R. solanacearum* from seed and planting materials by using PCR based techniques.

PCR Genomic assay	*Host*	*Detected from*	*Race/biovar of Bacteria*	*Reference*
SCAR primers				
Rep-PCR	Ginger, mioga and cucuma	–	*R. solanacearum* race 4	Horita *et al.*, 2004
RAPD	Brinjal, chilli and tomato	–	*R. solanacearum* race 3	James *et al.*, 2003
16S r RNA	Tomato	–	*R. solanacearum* race 1 biovar 3 and 4	Singh *et al.*, 2010.
PCR (RNA operon)				Pastrik *et al.*, 2002
Fli C	–	Soil	*R. solanacearum, R. pickettii*	Schonfeld *et al.*, 2003
	Geranium	Plant part	*R. solanacearum* race 3 biovar 2	Swanson *et al.*, 2007
Nested- PCR	Banana, tomato, chili, eggplant and tobacco	Soil	*R. solanacearum*	Khakvar *et al.*, 2008
hrp gene, PCRhrp gene, Nested- PCR	Tomato, potato tobacco, eggplant, pepper *Pelargonium asperum*	Plant and tuber	*R. solanacearum R. solanacearum* biovar 1, N2, 3, 4 & 5	Poussier *et al.*, 1999; Poussier *et al.*, 2000
Bio-PCR	Tomato, melon	Soil, Plant water	*R . solanacearum* race 1 biovar 3	Lin *et al.*, 2009; Singh *et al.*, 2014b
Co-operational PCR 16 S r RNA Co-oper ational PCR	*Pelargonium* spp	Cutting	*R. solanacearum* *R. solanacearum* race 3 biovar 2	Bertolini *et al.*, 2003 a; 2003b Macro- Noales *et al.*, 2008
PCR (insertion sequence)	Eight different host		*R. solanacearum* race 1	Lee *et al.*, 2001
16 r RNA, PCR16 r RNA, PCR16 r RNA, Multiplex PCR	– Potato	– Potato tuber	*R. solanacearum* subdivision 2a *R. solanacearum, R. solanacearum* biovar1, 2, N2, 3, 4 & 5	Boudazin *et al.*, 1999; Pastrik and Maiss, 2000; Seal *et al.*, 1999
PCR -RFLP	–	–	*R. solanacearum*	Poussier *et al.*, 1999; 2000
PCR-DGGE	–	–	*R. solanacearum*	Van Overbeek *et al.*, 2002

Contd...

Contd...

Multiplex–PCR (16S-23S r RNA ITS)	Potato	Tuber	*R. solanacearum*	Pastrik *et al.*, 2002; Bertolini *et al.*, 2003a)
Real–time PCR	Potato	Tuber and plant	*R. solanacearum*	Stead *et al.*, 2003; Weller *et al.*, 1999; Weller *et al.*, 2000; Ozakman and Achaad, 2003 Swanson *et al.*, 2007
Real Time Bio-PCR	Potato Ginger	Tuber and plant	*R. solanacearum* race 3 biovar 2 *R. solanacearum race 4*	
Real Time Bio-PCR	Geranium	Rhizomes plant	*R. solanacearum* race 3 biovar 2	
RNA based approaches (NASBA)			*R. solanacearum*	Betsink *et al.*, 2002
16S r (NASBA) RNA	Potato	Tuber, irrigation water	*R. solanacearum race 3 biovar2*	Van der Wolf *et al.*, 2004
IC-PCR	Tomato, pepper, weeds (*Physalis minima*, *Amaranthus spinosus* and *Euphorbia hirta)*	Plant and soil	*R. solanacearum race 1*	Dittapongpitch and Surat, 2003
Putative *Ipx* C gene	–	–	*R. solanacearm*	Villa *et al.*, 2003
Cytochromec1 signal peptide gene	Tomato	Soil and plants	*R. solanacearum* race 1 & 3 biovars 1, 2, 3 &4	Kang *et al.*, 2007

Co-operational PCR

It is a new PCR, which has high sensitivity for the amplification bacterial targets from plant material. The Co-PCR (co-operational amplification) technique can be performed easily in a simple reaction based on the simultaneous action of four or three primers. The reaction process consists of the simultaneous reverse transcription of two different fragments from the same target, one internal to the other, the production of four amplicons by the combination of the two pairs of primers, one pair external to the other, and the co-operational action of amplicons for the production of the largest fragment. Co-PCR usually produces the largest amplicon, in contrast to nested- PCR, which requires two sequential reactions and obtains the smallest fragment. Metal block and capillary air thermal cyclers have been employed for the detection of a bacterium, but by using only three primers (Caruso *et al.,* 2003), which shows the possibilities of this new approach. The low amount of reagents (ten times less than in conventional PCR) probably increases susceptibility to inhibitors. However, this step was not necessary when analyzing the presence of *R. solanacearum* in water (Caruso *et al.,* 2003). Co-PCR requires only one reaction, minimizing manipulation and reducing risk of contamination.

Real-time (Quantitative) PCR

Quantitative real time PCR has become possible by the development of detectors that can measure fluorescence that is emitted during the PCR cycle. This method is based on the 5'-3' exonuclease activity of the Taq DNA polymerase, which results in cleavage of fluorescent dye-labeled with different probes (TaqMan®) during PCR. The exponential nature of PCR in theory allows the amount of starting material to be calculated from the amount of product at any point in the reaction. In practice, however, reaction conditions can interfere with exponential amplification and affect product concentration. Early attempts at quantization involved stopping the PCR reaction at various points to generate standard curves, which resulted in a laborious, low-throughput process.

TaqMan probe

In this systems, an oligonucleotide probe sequence of approximately 25-30 nucleotides in length is labeled at the 5' end with a fluorochrome, usually 6-carboxyfluorescein (6-FAM) and a quencher fluorochrome, usually carboxytetramethyl-rhodamine (TAMRA), at the 3' end. The probe is degraded by the 5'-3' exonuclease activity of the Taq polymerase as it extends the primer during each PCR amplification cycle and the fluorescent chromophore released. The amount of fluorescence is monitored during amplification cycle and is propotional to the amount of PCR product generated. Real-time reaction monitoring with specific instruments and fluorescent probes combine amplification, detection and quantification in a single step. TaqMan probes consist of single-stranded oligonucleotides that are complementary to one of the target strands. A fluorescent dye adorns the 5' end and a quencher is bound to the 3' end. Fluorescence occurs when the polymerase replicates a template on which a TaqMan probe is bound and the 5' exonuclease activity cleaves the probe (Varma-Basil *et al.,* 2004). They have been proposed for detection of *R. solanacearum* (Weller *et al.,* 2000a; Ozakman and Schaad, 2003). Huang *et al*. (2009) detected *R. solanacearum* strain G 1000 from soil using specific primer pair R. sol 1-R. sol 2 and the TaqMan probe Rs-pro. The detection limit was 100 fg/µl

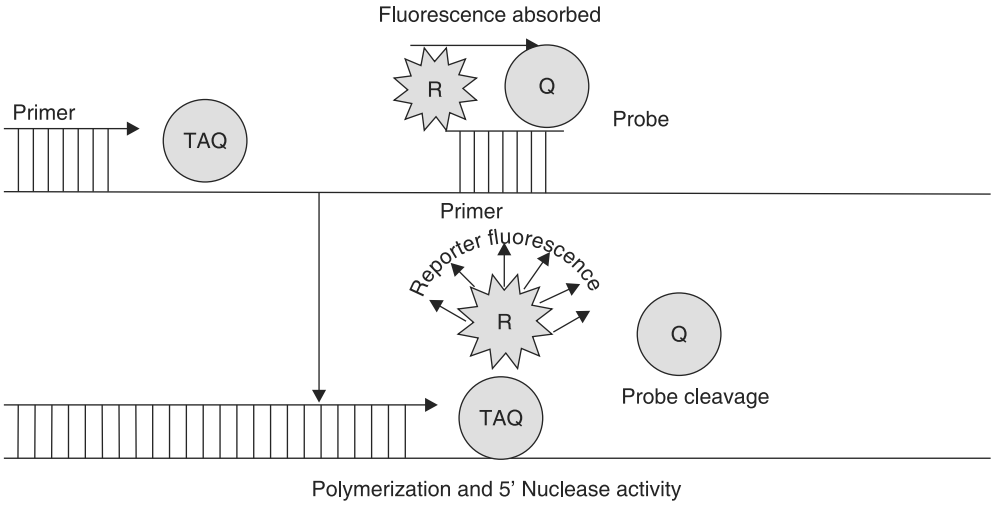

Fig. 2.4 TaqMan TM, chemistry in real-time PCR

Bio-PCR

In this PCR, combine the viable enrichment of mostly growth media with an enzymatic amplification, which is known as BIO-PCR. The target bacterium is enriched in liquid or solid media and detected at extremely low levels in seeds and other propagative materials. The BIO-PCR assay includes the following simple steps as (i) extracting a sample (ii) plating a sample onto agar media (half are retained for visual recovery or adding sample to liquid medium (iii) incubating for 15-72h, depending on the growth of target bacterium; (iv) washing plates to remove bacteria or centrifuge liquid medium and using 1 or 10 µl for direct PCR.

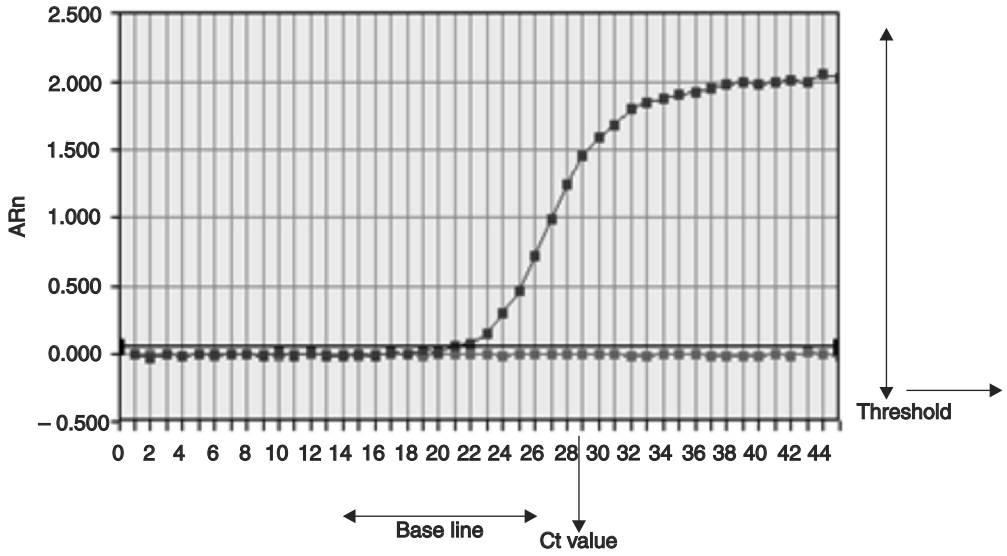

Fig. 2.5 Amplification plot from positive and negative real-time PCR reactions.

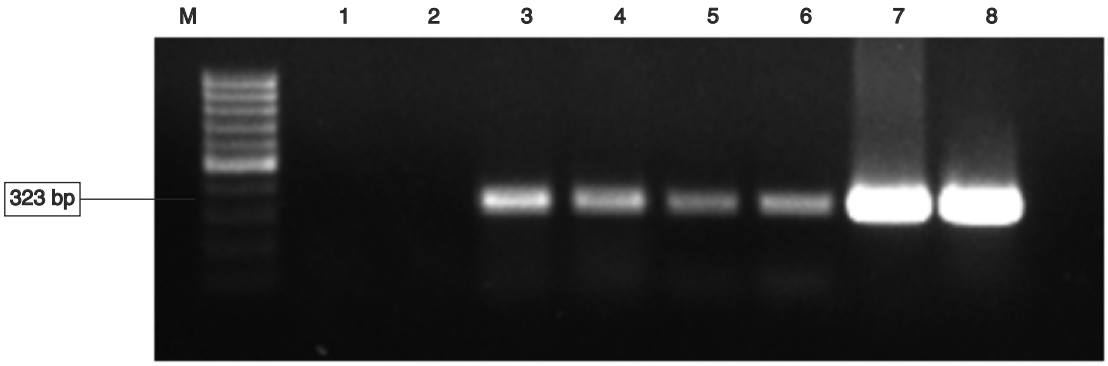

Fig. 2.6 Detection of *R. solanacearum* from irrigated water in farmer's tomato field by *hrp* gene based a set of primer hrp_allF and hrp_all R amplified at 323 bp. Lane M: 100 bp DNA ladder, lanes 1&2: soil samples, 3&4: water samples (BIO-PCR), 5&6: Direct irrigation water samples, 7: oozing from wilt infected tomato plant, 8: +ve control amplified.

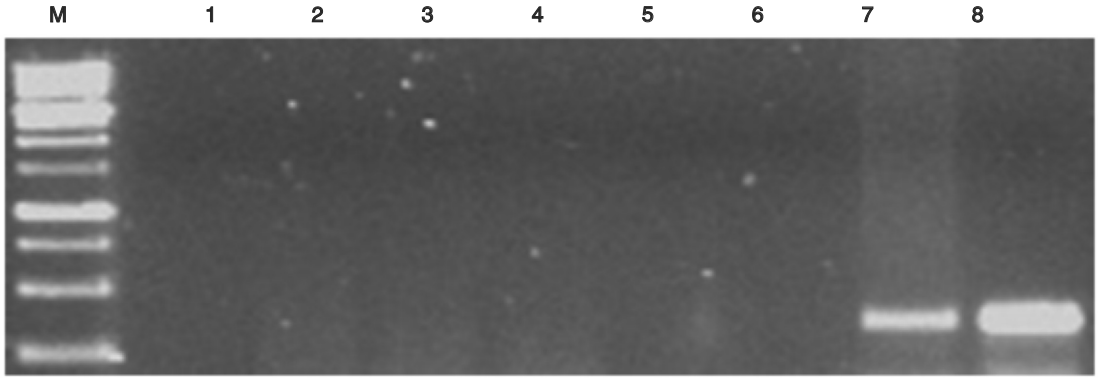

Fig. 2.7 Amplification of fragments of the *hrp* gene from *R. solanacearum* separated on an agarose gel showing the 323 bp. Lanes M: 1 Kb DNA ladder, 1-6: soil DNA, 5-6: direct water, 7: Bio culture from soil, 8: positive control

Multiplex PCR

Multiplex PCR allows the simultaneous and sensitive detection of different DNA targets in a single reaction. On the other hand, PCR detection protocols can be designed to verify the presence of more than one pathogen in plant material by looking for common specific sequences in two or more of them, or to detect related bacteria on multiple hosts. Multiplex PCR is useful in plant pathology because different bacteria and/or RNA viruses frequently infect a single host and consequently sensitive detection is needed for the propagation of pathogen-free plant material. There are several examples in plant pathology of simultaneous detection of several targets and the amplification by multiplex PCR.

Nested–PCR

Sensitivity and specificity problems associated with conventional PCR and RT-PCR can be reduced by using nested PCR-based methods, based on two consecutive rounds of amplification

initially developed for DNA it can be adapted to amplify RNA (RT-LAMP) (Fukuta *et al*., 2003). Kubota *et al*. (2008) developed four sets of primers to replicate the gene coding for the flagellar subunit, Fli C and conditions for detection of *R. solanacearum* were optimized to complete in 60 min at 65°C. Magnesium pyrophosphate resulting from the amplification reaction could be detected optically as an increase in the solution turbidity, and the DNA products spread in a reproducible ladder – like banding pattern after electrophoresis in an agarose gel. This method is rapid but and sensitive and detection limit of this LAMP assay is between 10^4-10^6 cfu/ml.

Microarray Technology

Microarray technology used for detection of bacterial plant pathogens is very limited. Microarrays are generally composed of thousands of specific probes spotted onto a solid surface either nylon or glass and each probe is complementary to a specific DNA sequence like genes, ITS, ribosomal DNA. Hybridization with the labeled complementary sequence provides a signal that can be detected and analyzed. Although there is great potential for microarray technology in the diagnosis of plant diseases, the practical development of this application is still in progress. For example, following the methodology utilized for genetic analysis (Brown and Botstein, 1999) large numbers of DNA probes used in two-dimensional arrays have allowed thousands of hybridization reactions to be analyzed at the same time (Hadidi *et al.,* 2004). Until now, the microarray technology focuses its use in multiplex format of similar or very different pathogens, taking advantage of the number of probes that can be employed in one chip (Bonants *et al.,* 2002; Schoen *et al*., 2002, 2003; Fessehaie *et al*., 2003; Franke-Whittle *et al*., 2005; Bonants *et al.,* 2005; Boonham *et al.,* 2007; van Doorn *et al.,* 2007; Pasquini *et al*., 2008). With the availability of genomic sequences of pathogens and the rapid development of microarray technology, as well as a renewed emphasis on detection and characterization of quarantine pathogens, there is a rush in the European Union to set up this technology and apply it to detection. The probes can be prepared in at least three basic formats: a) PCR fragments arrayed on nylon membranes, hybridized against cDNA samples radioactively labeled, called microarrays (Richmond *et al.,* 1999); b) PCR products spotted onto glass slides and DNA labeled with fluorescent dyes (Richmond *et al.,* 1999; Zimmer *et al.,* 2000; Wei *et al.,* 2001); and c) oligonucleotides of different lengths (from 18 to 70 bp) arrayed and hybridized with the same type of labelled DNA material (Lockhart *et al.,* 1996; Loy *et al.,* 2002, 2005; Fessehaie *et al.,* 2003; Peplies *et al.,* 2003). For bacterial detection, the material spotted until now is almost universally oligonucleotides targeting the 16S- 23S rDNA genes (Crocetti *et al.* 2000; Loy *et al.* 2002; Fessehaie *et al.,* 2003; Peplies *et al.,* 2003; Loy *et al.,* 2005; Franke-Whittle *et al*., 2005). The microarrays are analysed either by scanning or by a direct imaging system. Another type of microarray under development is called the nanochip (Sosnowski *et al.* 1997; Nanogen, Inc., San Diego, CA 92121, USA) based on an electronically addressable electrode array that provides direct electric field control over the transport of charged molecules to selected micro locations and concentration over an immobilized substrate. A particular feature of this system is that biotinylated immobilized molecules can be either oligo capture probes or amplified PCR samples. Hybridization is detected and analyzed by fluorescent oligo probes. By regulating the electric-field strength, hybridization stringency can be adjusted for homologous interactions. Nano chips have shown high specificity and accuracy to diagnose bacterial pathogens affecting

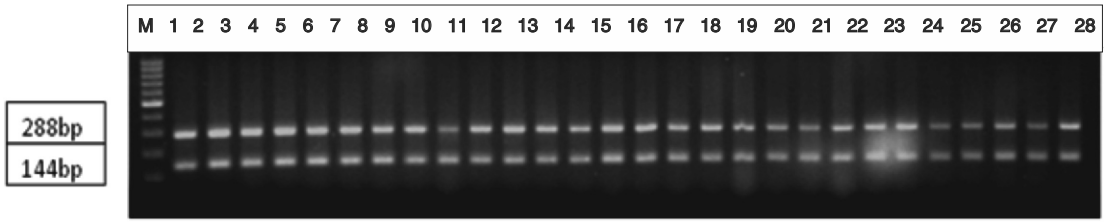

Fig. 2.9 130 isolates of *Ralstonia solanacearum* isolated from different crops of solanaceous crops (tomato, capsicum, chilli, brinjal and potato) from states of Jharkhand, Orissa, West Bengal, Himachal Pradesh, Uttarakhand and Jammu & Kashmir. All isolates of *R. solanacearum* belong to phylotype I, which is mostly dominated in Asian countries.

of microorganism. It is basically assumed that the higher the homology is between two strains, the more closely related the strains are in terms of evolution. These types of studies are known as phylogenetic studies. A sequevar or sequevance variant is defined as a group of strains with a highly conserved sequence within the area sequenced sequevars. A phylotype is defined as a group of strains that are closely related based on phylogenetic analysis of sequence data. Phylotypes were identified within the species that broadly reflects the ancestral relationships and geographical origins of the strains *In R. solanacearum* four phylotypes *viz*., Phylotype I, Phylotype II, Phylotype III and Phylotype IV have been reported by Fegan and Prior (2005). Each phylotype is composed of a number of sequevars (Fig. 2.10).

Amplified Fragment Length Polymorphism (AFLP) - PCR

The AFLP fingerprinting method is usually applied for discrimination at low taxonomic level, *i.e.*, species and strain level. Basically, genomic DNA from a sample is digested to completion, typically with two different restriction enzymes in order to produce a large number of fragments. Specific nucleotide adapters of 25-30 bp are ligated to the restricted DNA fragments. Oligonucleotide primers that anneal to these adapters are used, however, to impart additionally selectivity, the primers vary at their 3' end, such that they will amplify only a subset of the restricted DNA fragments. These 3'- end primer extensions must match the target sequence for amplification to occur. The amplified DNAs are are separated on a denaturing polyacrylamide gel and the amplified fragments are visualized by autoradiography. This method is highly discriminative and very useful in population studies, but expensive and labour intensive (Rademaker *et al*., 2000). AFLP methodology has already been used to study the diversity of race 3 isolates of *R. solanacearum* (Van der Wolf *et al*., 1998) but has never been used to analyse a worldwide collection of *R. solanacearum* strains. Although AFLP analysis cannot be performed on mixed cultures, such studies of pathogen diversity using pure cultures of representative strains will eventually permit rapid identification of unknown isolates and potentially be useful in diagnostics. Pousser *et al.* (2000) used AFLP method to analyse 96 *R. solanacearum* strains, one BDB strain and one *Ralstonia pickettii* strain collected from worldwide as described by Janssen *et al*. (1996) and Vos *et al*. (1995) with slight modifications. They used *Msp*I and *Sac*I to digest DNA instead of *Eco*RI and *Mse*I. Genomic DNA (200 ng per sample) was digested for 2 h at 37°C in 50 µl

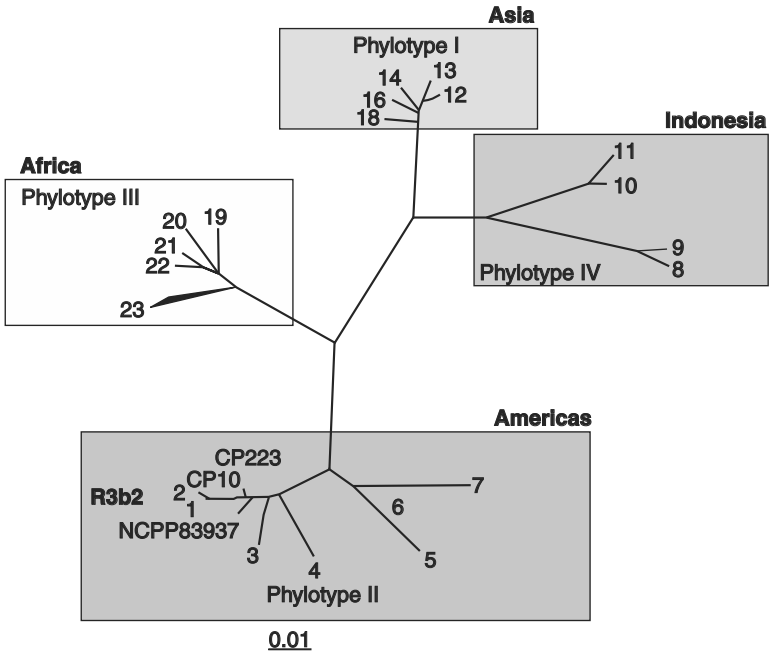

Fig. 2.10. Classification and geographic origins of *R. solanacearum* strains based on sequence analysis of endoglucanase is a type of cellulose, which is class of enzymes produced by fungi, bacteria and protozoans that catalyze the cellulolysis of endoglucanase gene sequences. Numbers indicate sequevars (1 to 23) *(Source: Fegan and Prior, 2005)*

(final volume) containing 5 U *Msp*I, 5 U *Sac*I, 0.125 µl BSA (10 µg μl^{-1}) and 2.5 µl 10x`One Phor All ' buffer. Next, 50 pmol double-stranded *Msp*I-adapter (5'-GACGATGAGTCCTGAA-3', 5'-CGTTCAGGACTCATC-3') (50 pmol μl^{-1}), 5 pmol double- stranded *Sac*I-adapter (5'-CTCGTAGACTGCGTACAAGCT- 3', 5'-TGTACGCAGTCTAC-3') (5 pmol μl^{-1}) 1 µl ATP (10 mM), 1 U T4 DNA ligase (5 Uμl^{-1}) and 2.5 µl 10x 'One Phor All' buffer were added to the digested DNA and the ligation reactions were performed for 3 h at 20°C. Digested (D) and ligated (L) DNA were diluted (D) eightfold and the resulting DLD DNA was then stored at 4°C until used. Selective amplifications were done with two primers (*Msp*I-primer and *Sac*I-primer) complementary to the adapter sequences, and the *Msp*I and *Sac*I restriction sites respectively, with additional selective nucleotides at their 3'- ends (cytosine for the *Sac*I-primer and cytosine plus guanine for the *Msp*I-primer). The *Sac*I-primer was labelled with γ^{-33P} [10 µCi (370 kBq) per DNA amplification and T4 polynucleotide kinase. DNA amplifications were carried out in a 50 µl reaction mixture. DLD DNA (5 ll) was added to 45 µl mixture containing 5 µl 10x PCR buffer, 1.5 µl labeled *Sac*I-primer (50 µg μl^{-1}), 2 µl unlabelled *Msp*I-primer (30 µg μl^{-1}), 1.5 µl $MgCl_2$ (50 mM), 8 ll of each dNTP (1.25 mM) and 0.6 ll *Taq* polymerase (5 U μl^{-1}). Amplifications were performed with a thermocycler by using the following protocol: 30 cycles

At selected sampling points, collect water samples by filling disposable sterile tubes or bottles at a depth if possible below 30 cm and within 2 m from the bank. For processing and sewage effluents, collect samples from the point of effluent discharge. Sample sizes up to 500 ml per sampling point are recommended. If smaller samples are preferred, it is advisable to take samples on at least three occasions per sampling point, each sample consisting of two replicated sub-samples of at least 30 ml. For intensive survey work, select at least three sampling points per 3 km of watercourse and ensure that tributaries entering the watercourse are also sampled.

Transport samples in cool dark conditions (4 to 10°C) and test within 24 hours.

If required, the bacterial fraction may be concentrated using one of the following methods:

(a) Centrifuge 30 to 50 ml sub-samples at 10,000 g for 10 minutes (or 7000 g for 15 minutes) preferably at 4 to 10°C, discard the supernatant and resuspend the pellet in 1 ml pellet buffer (10 mM phosphate buffer, pH 7.2).

(b) Membrane filtration (minimum pore size 0.45 μm) followed by washing the filter in 5 to 10 ml pellet buffer and retention of the washings. This method is suitable for larger volumes of water containing low numbers of saprophytes. Concentration is usually not advisable for samples of potato processing or sewage effluent since increased populations of competing saprophytic bacteria will inhibit detection of *R. solanacearum*.

Detection and Identification of *R. solanacearum* in Soil

Principles

The validated detection scheme, described in this section, is applicable for pathogen detection in soil samples but can also be used to test samples of solid potato processing waste or sewage sludge. However, it should be noted that these methods are insufficiently sensitive to guarantee detection of low and/or irregularly dispersed populations of *R. solanacearum* that may occur in naturally infested samples of these substrates. The limitations in sensitivity of this test scheme should be considered when assessing the reliability of any negative results obtained and also when used in surveys to determine presence or absence of the pathogen in soils or sludges. The most reliable test for presence of the pathogen in a field soil is to plant a susceptible host and monitor it for infection, but even with this method low levels of contamination will escape detection.

Sample Preparation

Sampling of field soil should follow standard principles used for nematode sampling. Collect 0.5 to 1 kg of soil per sample from 60 sites per 0.3 ha from a depth of 10 to 20 cm (or in a grid of 7 × 7 metres) If the pathogen is suspected to be present, increase the number of collection points to 120 per 0.3 ha. Maintain samples at 12 to 15°C prior to testing. Sample potato processing and sewage sludges by collecting a total of 1 kg from sites are representing the total volume of sludge to be tested. Mix each sample well before testing. Disperse sub-samples of 10 to 25 g of soil or sludge by rotary shaking (250 rpm) in 60 to 150 ml extraction buffer for up to two hours. If required, addition of 0,02% sterile Tween-20 and 10 to 20 g sterile gravel may assist dispersion. Maintain the suspension at 4°C during testing.

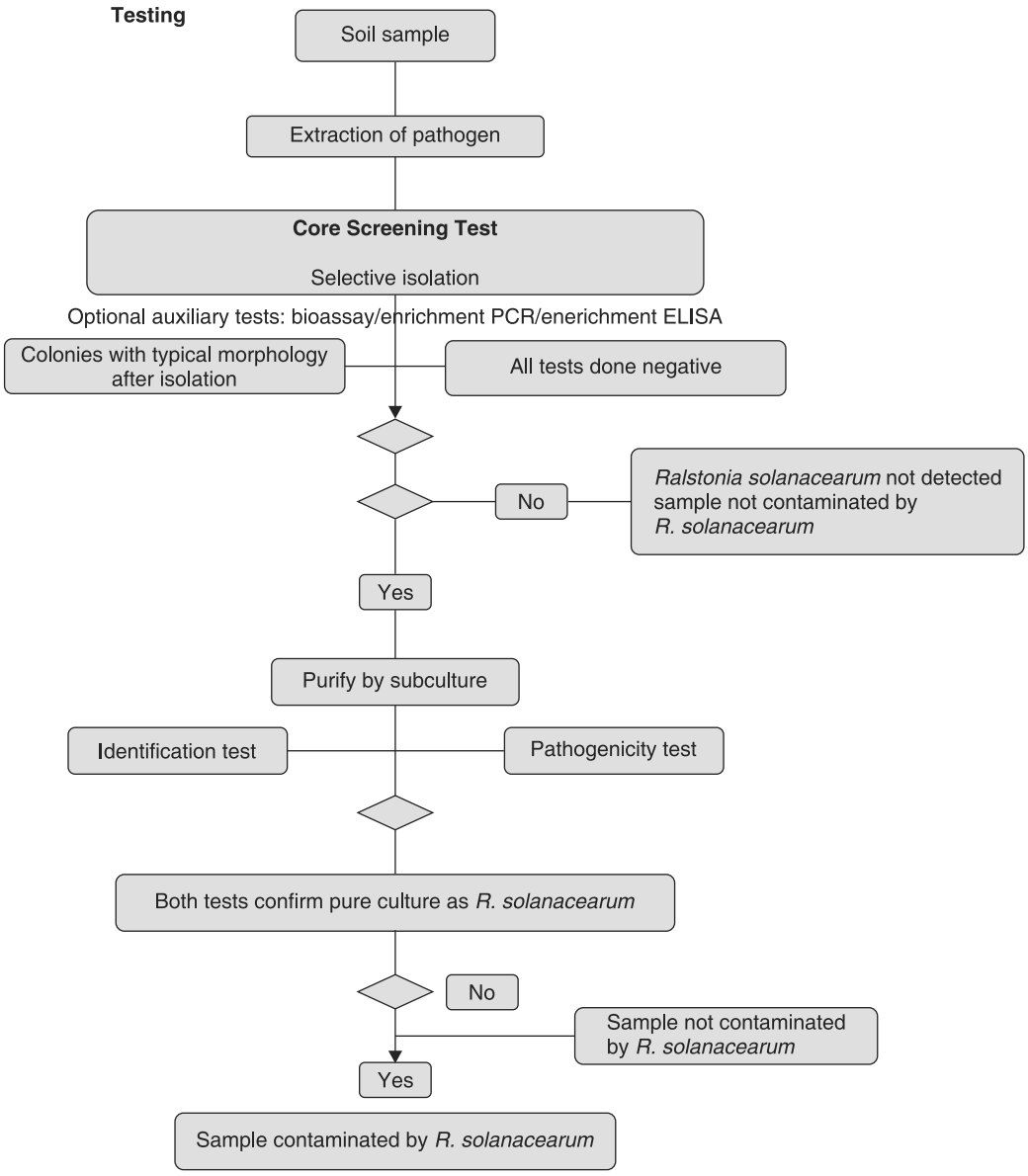

Fig. 2.12 Flow chart for detection and identification of *R. solanacearum* in soil