
***In vitro* screening of bioantagonistic agents and plant extracts to control bacterial wilt (*Ralstonia solanacearum*) of tomato (*Lycopersicon esculentum*)**

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Field survey was undertaken in major tomato growing districts of the Karnataka and other states in Southern Part of India to isolate bacterial wilt pathogen *Ralstonia solanacearum* from wilted tomato plants and soil samples. A total of 100 isolates named as RS1-RS100 were subjected to cultural, morphological, biochemical and pathogenicity studies. Significant variations existed among the isolates with respect to rate, type of growth and colony colour. According to the pathogenicity tests conducted, around 57% of the isolates were found to be highly virulent. The population build up of the pathogen on the root system was found to have played an important role in relation to the pattern of occurrence of pathological wilting among tomato plants in the field. Bacteria with the same characteristics as those inoculated were re isolated from the infected plants, uninoculated plants remained healthy.

Key words: Tomato, *R. solanacearum*, morphological studies, biochemical assay

Introduction

Bacterial wilt caused by *R. solanacearum* is deemed to be one of the most important plant diseases in tropical agriculture (Hayward 1990; Milling *et al.* 2011). It has a large host range of more than 200 species in 50 families (Aliye *et al.* 2008). These globally dispersed and heterogeneous strains cause bacterial wilt diseases, which have major socioeconomic impacts (Yabuuchi *et al.* 1995, Getachew *et al.* 2011). The disease affects a wide range of economically important crops such as tomato, potato, eggplant, chilli and non *Solanaceous* crops such as banana and groundnut in India (Anuratha *et al.* 1990). The disease is called southern bacterial blight, *R. solanaceous* wilt and other common names in countries where it occurs (Kelman *et al.* 1964).

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Bacterial wilt is said to be causing 15 to 55% crop losses around the world (Lukyanenko, 1991). In India, a study showed 10 to 100% incidence of bacterial wilt during the summer (Kishun, 1985). Infested soil and surface water, including irrigation water, are the primary sources of inoculum. The pathogen infects roots of susceptible plants, usually through wounds (Pradhanang *et al.* 2005). Colonization by the bacterium within the xylem prevents water movement into upper portion of the plant tissue (Kelman, 1998). The symptoms start as leaf drooping followed by wilting of whole plants within a few days, leading to total plant collapse. The infected plants may recover, temporarily, in the evening, when temperatures are cooler. A few days later, a sudden and permanent wilt occurs. The roots and lower portion of the stems have a browning of their vascular system. The invaded roots may rot due to infection from secondary bacteria (McCarter, 1991). *R. solanacearum* is a gram negative, rod shaped, strictly aerobic bacterium that is 0.5-0.7 x 1.5-2.0 µm in size, with a single polar flagellum. Individual bacterial colonies are usually visible after 36 to 48 hrs of growth at 28°C and colonies of the normal or virulent type are white or cream colored, irregularly shaped, highly fluidal and opaque. Occasionally colonies of the mutant or non virulent type appear uniformly round, smaller and butyrous or dry. A Kelman's selective nutrient tetrazolium chloride (TZC) medium (Kelman, 1954) can differentiate the two colony types on this medium. Strains of *R. solanacearum* have been classified into five biovars (He *et al.* 1983 and Kumar *et al.* 1993) and five races (Buddenhagen *et al.* 1962; Bin Li *et al.* 2010).

Biological control is acceptable as a key practice in sustainable agriculture (Azcon Augiler and Barea, 1996). Biological control preserves environmental quality by reducing the dependency on chemical input and maintaining sustainable management practices (Barea and Jeffries, 1995). Alternative methods of disease control are highly desirable due to increasing demand for the safety of agricultural produces, occurrence of fungicide resistant pathogen populations, and environmental problems caused by fungicide residues. Plant growth promoting bacteria (PGPR) strains and *Trichoderma* species were reported to be a promising bio-control agent to control *R. solanacearum*. It was found that they were able to reduce the disease in different levels and increased the yield of tomato plant (Guo *et al.* 2004). The aim of the present study was to isolate and characterize bacterial wilt pathogen from various agroclimatic regions and screen for potential antagonists for its biocontrol.

Materials and methods

Field survey and Sample collection

Field survey was conducted to determine the prevalence of bacterial wilt in tomato plants in major tomato growing districts of India (Bangalore Rural, Mysore, Mandya, Kolar, Chikkaballapur, Tumkur, Hassan, and Shimoga) of Karnataka and Tamil Nadu (Vellore and Hosur), Andhra Pradesh (Chittoor and Anantapur), Maharashtra (Pune). The total number of healthy and wilted plants was counted in a 1 m² area and wilt percentage incidence was recorded. The plants were observed for the typical symptoms of bacterial wilt viz., leaf yellowing, wilting and vascular browning (Figure.1). The plant and rhizosphere soil samples were collected.



Fig. 1. Brownish discoloration of vascular tissue due to bacterial wilt

Isolation of pathogen from soil and plant samples

The plant materials were surface sterilized with 1% NaOCl solution for 1 to 2 min, followed by three repeated washings with distilled water and blot dried. Then the plant sections (0.5–1 cm) were plated onto 2, 3, 5 Triphenyl Tetrazolium chloride (Kelman's TZC agar) medium (Kelman, 1954). The plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 24–48 h. Isolation from rhizosphere soil samples was done by dilution plate technique on modified semi selective medium, South Africa (SMSA) agar medium (Elphinstone *et al.* 1996).

R. solanacearum was isolated from a freshly wilted tomato plant by streaking a loopful of flowing ooze containing the bacteria onto sterile TZC agar plates (Danks *et al.* 2000). After isolation, they were stored in sterile distilled water at room temperature in sterile polypropylene tubes (Kelman and Person, 1961). Glycerol stocks of the different isolates were made and stored at -80°C for long term preservation of the culture. The strains were subjected to different biochemical, physiological, hypersensitive and pathogenicity tests for confirmation of the identity of the pathogen (Vanitha *et al.* 2009).

Identification of isolates

The identification of the bacteria isolated from tomato plants based on the morphological, physiological, cultural, biochemical and pathogenicity studies were carried out, as characterized by Kelman (1954) and Schaad (1992). Morphological, cultural and physiological characterization: All the isolates were grown on TZC plates at $28 \pm 2^{\circ}\text{C}$ for 48hr (Fegan and Prior, 2005; Hayward, 1991). Observation was made on colony colour, shape, size, surface, margin, elevation, opacity, consistency, gram reaction and motility (Hayward, 1964). Temperature tolerance was tested by growing the isolates at 37 and 41°C (Vanitha *et al.* 2008). Sodium chloride tolerance was tested according to Sands (1990).

According to Hayward (1964) who characterize the isolates of *R. solanacearum* into biovars on the basis of oxidation of carbohydrates, biovars of *R. solanacearum* were differentiated according to their ability to oxidize disaccharides and hexose alcohols as seen in Table. 1.

Table 1. Biovar characterization of *R. solanacearum* strains

Biovar	Utilization of carbohydrates					
	1	2	3	4	5	6
	Lactose	Maltose	Cellobiose	Mannitol	Sorbitol	Dulcitol
I	-	-	-	-	-	-
II	+	+	+	-	-	-
III	+	+	+	+	+	+
IV	-	-	-	+	+	+
V	+	+	+	+	-	-

Note: + Utilization of sugars, - Not utilization of sugars

Biochemical characterization: subjecting the isolated bacterial colonies to various biochemical tests; gram's staining, poly β -hydroxyl butyrate- PHB granules test (Lelliott and Stead 1987), KOH solubility test (Fahy and Hayward 1983), oxidase test (Hildebrand and Senroth, 1972 and Sands, 1990), gelatin hydrolysis, starch hydrolysis, nitrate reduction (Fahy and Persley, 1983), arginine dihydrolase activity, catalase, H_2S production, citrate utilization (Hildebrand *et al.* 1988), triple sugar iron agar test, pectin hydrolysis, tween 80 hydrolysis, urease test, indole production, levan production (Vanitha *et al.* 2008).

Pathogenicity testing of isolates

Virulence analysis of the isolates was carried out on a set of three tomato cultivars, Arka Meghali, Arka Saurabha and Arka Vikas which are susceptible to bacterial wilt. The 20 days old healthy seedlings were selected and used for further pathogenicity assay. Bacterial inoculum was prepared in sucrose peptone broth (Hendrick *et al.* 1984, Mitsuo *et al.* 2004) and pelleted by centrifuging at 12,000 rpm for 10 min, suspensions were prepared in sterile distilled water and spectrophotometrically adjusted to OD_{600nm} = 0.1 (approximately 10⁸ CFU per ml) (Ran *et al.* 2005). Root dip method: the roots were trimmed with a sterile scissor and submerged in the bacterial suspension for 30 mins (Prior and Steva, 1990). The inoculated seedlings were transplanted to mini pots containing soil and sand in 1:1 ratio and incubated in greenhouse. The day and night temperatures varied between 25-35⁰ C with 12h light and 12h dark (Klement *et al.* 1990). Soil drenching method: the root system of each plant was wounded with a scalpel; 5 ml of inoculum per plant was poured on the wounded root system. Plants were placed in a greenhouse and observed daily (Williamson *et al.* 2002). Each experiment was repeated twice, with two replications of 10 plants for each treatment. Observations were made from one week after inoculation. If the plant showed typical wilt symptoms, the interaction was considered as pathogenic. The isolates were categorized into 4 group's viz., highly pathogenic, moderately pathogenic, weakly pathogenic and non pathogenic based on the symptomatological variations in the test tomato varieties, whereas non inoculated tomato seedlings showed no symptoms and hence served as control (Veerapaneni *et al.* 1997; Tans *et al.* 2001).

In vitro selection of antagonistic Pseudomonas fluorescens and Trichoderma species against R. solanacearum.

Isolation and selection of bioantagonistic of Pseudomonas fluorescens

Isolation was carried out according to the methods described by Labeda (1990). The potential bioantagonistic bacteria were isolated from rhizoplane soil of healthy tomato plants by soil dilution method using King's B medium and incubated at 28 ± 2⁰C for two days and maintained on Kings B slants by regular sub culturing. The identity of the *P. fluorescens* strains were confirmed by morphological, cultural and biochemical tests (Rekha *et al.* 2010). Among twenty *P. fluorescens* strains, only ten strains were selected for further study. The *R. solanacearum* suspensions were adjusted to 10⁸ cfu per ml and swabbed on TSA (Tryptic Soy Agar). The swabbed plates were spot inoculated with *P. fluorescens* strains and incubated. Following incubation, the zone of inhibition

was observed. There were four replicates for each treatment. Isolation and selection of bioantagonistic *Trichoderma* species: *Trichoderma* strains were isolated from rhizoplane soil of healthy tomato plants by standard soil dilution method on PDA (Potato Dextrose Agar) at 25± 2°C. Pure cultures of the *Trichoderma* isolates were maintained on PDA and identified using cultural and morphological characters (Watts *et al.* 1988). The reidentification of the strains was done by National Fungal Culture collection of India (NFCCI), Agharkar Research Institute, Pune.

Antagonistic activity of *Trichoderma* spp. which was tested against ten highly virulent strains of *R. solanacearum* by *in vitro* techniques using TSA (Ran *et al.* 2005). 100µl supernatants from one week old culture broths of *Trichoderma* grown in Potato Dextrose Broth (PDB) were tested by well diffusion method (Kamal *et al.* 2008). Following incubation, the zone of inhibition was observed. There were four replicates for each treatment.

Antibacterial activity of plant extracts against R. solanacearum.

The antibacterial activity of crude plant extracts of Gulancha tinospora (*Tinospora cordifolia*), Coriander (*Coriandrum sativum*), Mustard (*Brassica alba*), Neem (*Azadirachta Indica*), Lemon plant (*Citrus lemonium*), Lemon grass (*Cymbopogon flexuosus*), Peppermint (*Mentha piperita*), Guava (*Psidium guajava*), Papaya (*Carica papaya*), Turmeric (*Curcuma longa*), Pomogranate (*Punica granatum*) were tested against *R. solanacearum*. The crude extracts were prepared from different plant parts according to the method described by Terblanche and de Villiers (1998). Antibacterial activity was tested against the pathogen by Agar well diffusion method (Shrisha *et al.* 2011). Following incubation, the zone of inhibition was observed. There were four replicates for each treatment.

Results

Field survey and sample collection

The farmer fields surveyed in different districts of Karnataka, India for bacterial wilt incidence of tomato are depicted in Figure 2.

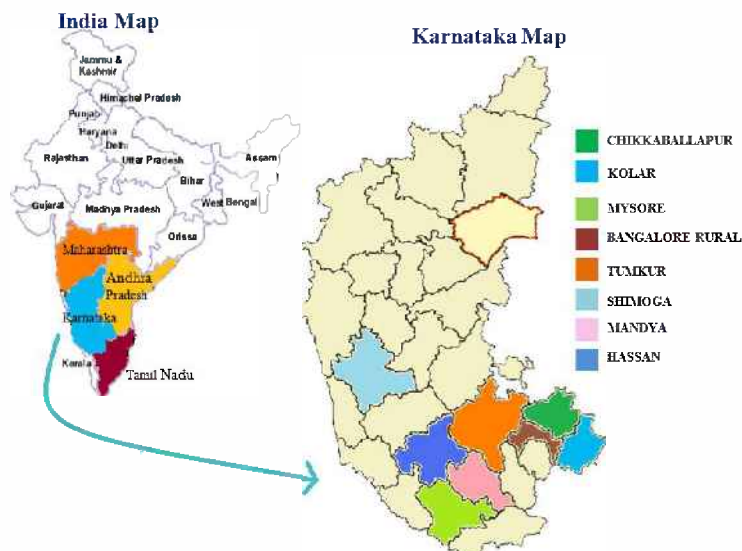


Fig. 2. Map depicting different states of India and different districts of Karnataka, the location of the current studies of field survey for the bacterial wilt incidence.

Based on the field survey in major tomato growing areas of Karnataka, Tamil Nadu, Andhra Pradesh and Maharashtra, it was evident that the incidence of *R. solanacearum* was highly prevalent. In the rating scale, highest disease incidence in tomato was recorded in Chikkaballapur, Kolar, Mysore, Bangalore Rural, Tumkur Shimoga, and Mandya, districts of Karnataka. Fields of Chittoor, Anantapur, Vellore, Hosur, Pune, were also affected by the disease.

Isolation of R. solanacearum

After incubation white fluidal colonies with pink centres were observed in soil dilution plates and around the pieces of plant material on the isolation plates. A total of 100 strains of *R. solanacearum* were isolated, identified and stored as pure cultures on TZC slants at 4⁰C for further studies.

Identification of isolates

Stem streaming method

The threads of bacterial ooze exuding from the infected xylem vascular bundles were observed from wilted plants (Figure. 3). This streaming test was a valuable diagnostic tool for quick detection of bacterial wilt in the field (Allen *et al.* 2001).



Figure 3. Cross section of the infected stem showing bacterial streaming

Morphological, Microscopic and physiological identification

Virulent isolates grown on TZC medium (Engelbrecht, 1994) were highly fluidal, white colored with a light pink centre (Figure. 4) and round to irregular margin, 7.0-9.0 mm diameter. On the other hand, the avirulent colonies were round, deep red color with narrow bluish border (Jeffrey B. Jones *et al.*, 2008; Partrice G. Champoiseau *et al.* 2008). Microscopic studies revealed that bacterial isolates were gram-negative, rod-shaped, 0.5-0.7 x 1.5-2.0 μm in size non-capsulated and non-spore forming, strictly aerobic bacterium. The pathogen was tolerant to sodium chloride concentrations up to 2% beyond which growth was inhibited. All strains were grown at 37°C and failed at 40°C. Motility of each isolate was confirmed by performing hanging drop method (Hayward, AC. 1964).



Fig. 4. Pink centered virulent colonies of *R. solanacearum* on TZC agar medium.

Characterization of R. solanacearum isolates into biovars based on oxidation of carbohydrates as per Hayward's classification system

Out of 100 isolates, 85 isolates oxidized and utilized all sugar alcohols and disaccharides the 15 isolates from Karnataka utilized the three disaccharides and two alcoholic sugars except one alcoholic sugar (dulcitol). From the observation it was concluded that the 85 isolates from different agroclimatic regions of Karnataka and other parts of India belong to biovar III.

Biochemical characterization

The strains were characterized biochemically and results tabulated as seen in Table 2.

Table 2. Biochemical and physiological characteristics of *R. solanacearum* from wilted tomato plant in Karnataka and other parts of India compared with authentic strain *R. solanacearum*

SL.No.	Biochemical/ Physiological Tests	<i>R. solanacearum</i> isolates
1	Gram's Reaction	-
2	Motility	+
3	Oxidase	+
4	Catalase	+
5	Indole production	-
6	Non-Gram staining or KOH solubility	+
7	Chromogenesis	-
8	Action on Litmus milk	+
9	Poly-β-Hydroxy butyrate accumulation	+
10	Citrate utilization test	+
11	triple sugar iron agar	+
12	Hydrolysis of Tween 80	W
13	Gelatin hydrolysis	-
14	Esculin hydrolysis	-
15	Starch hydrolysis	-
16	H ₂ S production	+
17	Levin Formation	+
18	Nitrate reduction:	+
19	Urease	-
20	Fermentative	-
21	Arginine dihydrolase	W
22	Growth at 41 °C	-
23	Salt tolerance at 1%	+
24	Salt tolerance at 2%	-
25	Fluorescent on KB medium	-

All tests were conducted in four replicates and were repeated thrice; '+' indicates positive reaction; '-' indicates negative reaction and 'W' indicates weak reaction

Pathogenicity testing of R. solanacearum

Pathogenicity was confirmed by the development of wilt symptoms on test plants after 7 days of inoculation followed by reisolation and identification of the causal organism from diseased plants (Elphinstone *et al.*, 1998). Based on the development of visible symptoms, *R. solanacearum* strains were grouped into highly pathogenic, moderately pathogenic, weakly pathogenic and avirulent (Figure 5). The results showed that 57 isolates were highly virulent and induced complete wilting after 25 days of inoculation. 31 isolates induced 70–80% wilt, while 7 isolates were least virulent inducing only 50% wilting after the same period. 5 Isolates failed to cause any disease in the infected plants and plant remained healthy without any disease symptoms. (Figure 6).



Fig. 5. The symptomatic variation on tomato plants of 1-5 scale studied under green house conditions. Symptom 1 to 5.
1-No symptoms, 2- Slight chlorosis, 3- Moderate chlorosis, 4- Severe chlorosis, 5- Death of the plant.

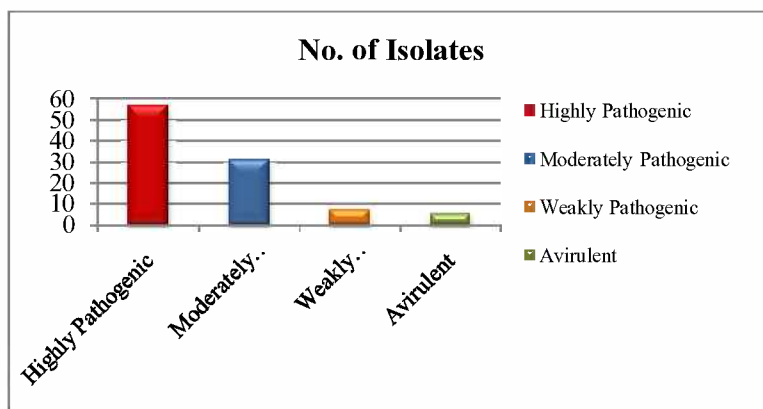


Fig. 6. Grouping of *R. solanacearum* isolates based on pathogenicity variation.

In vitro* selection of antagonistic *Pseudomonas fluorescens* and *Trichoderma* species against *R. solanacearum

All 10 strains of *P. fluorescens* tested showed antagonistic effects against *R. solanacearum*, with inhibition zone radii ranging from 3 to 29mm (Figure. 7). *P. fluorescens* strain 5 was most potent inhibiting all test pathogen strains, followed by Pf 4, Pf 6, and Pf 9. However the activity of other *P. fluorescens* isolates varied considerably against different pathogen isolates (Figure. 10).

Results obtained the antagonistic fungi of *Trichoderma viride*, *T. harzianum*, *T. asperellum*, *T. flavofuscum* and *T. koningii*.

Among the *Trichoderma* sp. tested, against ten strains of *R. solanacearum*, *T. asperellum* showed most potent inhibiting all test pathogen strains, followed by *T. viride*, *T. harzianum* (Figure. 8). However the activity of other *Trichoderma* isolates varied considerably against different pathogen isolates (Figure. 11).

The plant extracts were tested against ten *R. solanacearum* strains and the results were observed (Figure. 9). Among 12 tested plant extracts against ten strains of *R. solanacearum*, Neem and Pomegranate showed greater inhibition, and Guava, Pappaya, Turmeric and Gulancha tinospora showed relatively higher level of inhibition whereas Japanese mint and Mustard showed relatively lower level of inhibition activity (Figure. 12).



Fig. 7. Antagonistic activity of *Pseudomonas fluorescens* against *R. solanacearum*.

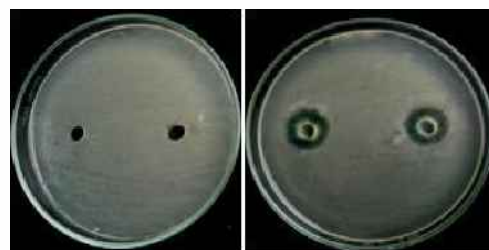


Fig. 8. Antagonistic activity of *Trichoderma asperellum* against *R. solanacearum*.

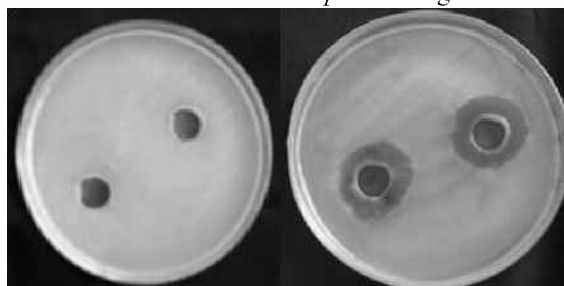


Fig. 9. Antibacterial activity of Plant extracts against *R. solanacearum*.

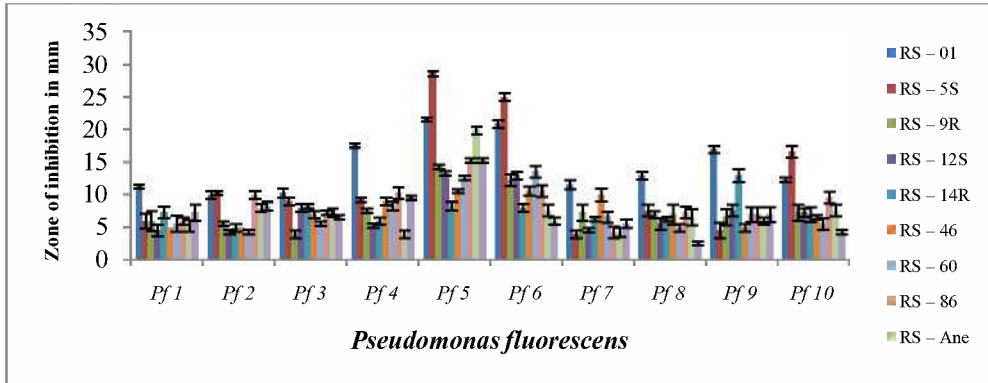


Fig. 10. Effect of different isolates of *Pseudomonas fluorescens* against growth of *R. solanacearum*.

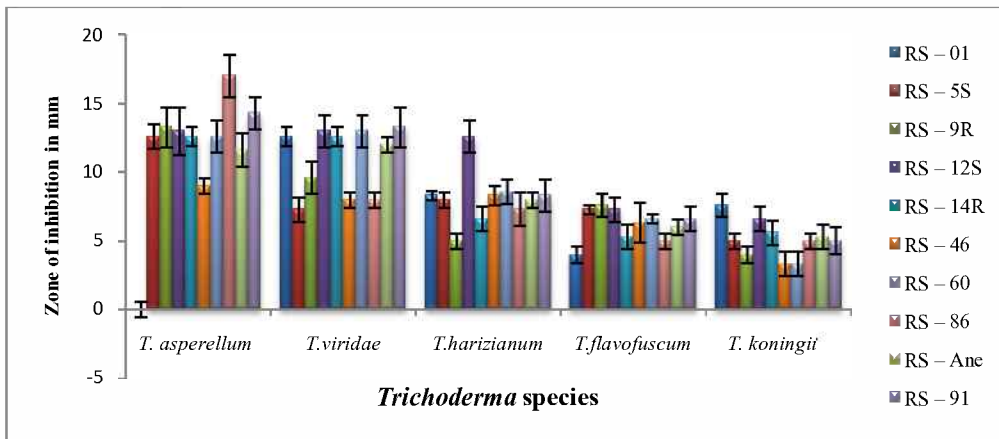


Fig. 11. Effect of different isolates of *Trichoderma* species against growth of *R. solanacearum*.

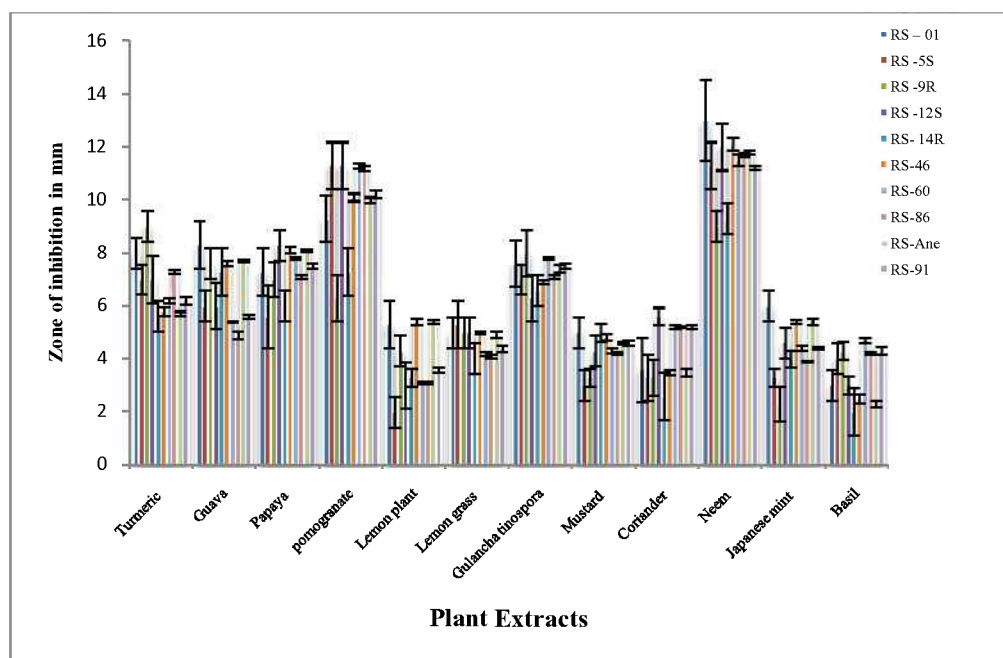


Fig. 12. Efficacy of plant extracts against *R. solanacearum* (zone of inhibition in mm).

Discussions

Field survey revealed the prevalence of the disease in different parts of Karnataka, Andhra Pradesh, Maharashtra, Tamil Nadu, Kerala and Orissa. The bacterial wilt pathogen *R. solanacearum* was commonly associated with diseased plants and soil samples collected from the infected fields. The high incidence of the disease in Karnataka indicates that it is a recurrent problem in the tomato growing areas and very less or no attempts were made to isolate the pathogens, identify and characterize them unless symptoms appeared. The bacterial wilt was differentiated from fungal wilt by bacterial ooze in stem streaming method. The use of modified SMSA medium with antibiotics allowed the differentiation of virulent and non virulent strains among the isolated strains. In the present study, *R. solanacearum* was easily detected by pour plate, spread plate and direct plating methods on the modified TZC medium. The presence of *R. solanacearum* on any part of the plant can serve as the potential source of inoculums and plays a very important role in spreading the bacterial wilt of tomato across many fields thus affecting the yield and decreasing the economy of the tomato growers.

Microscopic Observations clearly indicated that the organism was gram negative and thus the preliminary identification of *R. solanacearum* was

confirmed. The morphological and cultural characteristics on specific TZC agar medium, physiological, biochemical characterization, pathogenicity test results (Kelman, 1954) confirmed the identity of the isolates. The utilization of different carbohydrate sources by the isolates was same as that of Hayward's results (1964) and they were characterized into Biovars.

According to Koch's postulates, the pathogenicity tests were conducted to check the potential of the isolates. The soil drenching method yielded good results and was a better method for pathogenicity assay compared to the root dip method. The above test results indicated that the pathogen is highly potential and is spread widely across different parts of Karnataka, hampering net yield of the tomato production across the state. The *in vitro* studies have shown that *Pseudomonas fluorescens*, *Trichoderma* species and plant extracts have potential antibacterial activity against the pathogen *R. solanacearum*. Hence the further study is necessary to check the ability of the biocontrol agents to control the disease under field conditions and hence develop environmental friendly, integrated pest management methods.

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