MOLECULAR CHARACTERIZATION AND SERODIAGNOSIS OF VASCULAR PATHOGENS AFFECTING TOMATO

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LIST OF ABBREVIATIONS

%	: percentage
µg	: micro gram
µl	: micro liter
AP	: affinity purified
cm	: centimeter
ELISA	: enzyme linked immuno sorbent assay
g	: gram
h	: hour
IAA	: indole acetic acid
IgG	: immono globulins
kg	: kilogram
m	: meter
mg	: milli gram
mm	: millimeter
ml	: milliliter
mM	: mili molar
ng	: nano gram
No.	: number
^{o}C	: degree celesius
OD	: optical density
PCR	: polymerase chain reaction
PBS	: phosphate buffer saline
PDA	: potato dextrose agar
pH	: hydrogen ion concentration
pM	: pico molar
PNPP	: p-nitro phenyl phosphate
RAPD	: random amplified polymorphic DNA
SAHN	: sequential agglomerative hierarchal nested
SDS	: sodium dodyecyl sulphate
Taq	: thermos aquaticus
TZC	: triphenyl tetrazolium chloride
U	: unit
UPGMA	: unweighted pair group arithmetic mean method
UV	: ultra violate

1. INTRODUCTION

Tomato (Solanum lycopersicum, formerly Lycopersicon esculentum Mill.) is one of the most widely grown vegetable crops in the world. It is used as a fresh vegetable and can also be processed and canned as a paste, juice, sauce, powder or as a whole (Barone and Frusciante, 2007). The popularity of tomato is rising among consumers, not only because of its good taste, but also because it contains high levels of vitamin C, lycopene, and beta-carotene, which are anti-oxidants that promote good health. The high demand for tomato makes it a high value crop that can generate much income for farmers. Tomato is best adapted to warm and dry environments, but during the hot-wet season, yields are low due to poor fruit-setting caused by the high temperatures, as well as many severe disease problems.

Tomato cultivation has become more popular since the mid nineteenth century because of its varied climatic tolerance and high nutritive value. The area under tomato cultivation in the world is about 35.42 lakh hectares with an annual production of 951.27 lakh tonnes (Anon., 1999). In India tomato occupies an area of about 534.5 thousand hectares with a production of about 9361.8 thousand million tonnes. The crop occupies an area of about 44.5 thousand hectares in Karnataka, accounting for 1142 million tonnes of fruits (Anon., 2008).

Tomato crop is attacked by various plant pathogens. Fusarial wilt of tomato caused by Fusarium oxysporum f. sp. lycopersici (Sacc.) W. C. Synder and H. N. Hansen is an economically important disease and is a destructive disease of tomato crop worldwide (Jones et al., 1991). The disease is further aggravated in presence of a plant nematode Meloidogyne incognita (Kofoid and White), the latter alone causing root knot disease. Another vascular wilt caused by a bacterium Ralstonia solanacearum (Pseudomonas solanacearum) is one of the most important diseases (which can also interact with root knot nematodes) on agricultural crops, particularly on horticultural crops. Yield losses due to the disease on different crops varies and is difficult to measure accurately, but were estimated at 15-35% on tomato (Hayward, 1994; CAB International, 2004). All these together are limiting factors in tomato production throughout Asia.

The range of techniques available for identification and monitoring of soil-borne pathogens has expanded over past decades including immunological assays and most recently, nucleic acid hybridization and related nucleic acid techniques to identify and track soil borne plant pathogens in soil and plant tissues.

Conventional methods of culturing and microscopic analysis are time consuming in disease management. Many soil borne pathogens multiply and proliferate without producing disease symptoms and in such cases their detection and monitoring are vital in preventing build up of populations of such soil-borne pathogens, reaching economic threshold levels. Early disease diagnoses are important steps for a successful disease control. The first important step in the disease diagnosis is accurate detection of the pathogen. This needs to be done using an effective and efficient technique that enables to control the disease effectively, quickly, and accurately.

Therefore in recent years, research has been directed towards developing rapid, sensitive and specific diagnostic assays to detect the presence of soil borne pathogens in plant and soil samples. New molecular techniques, such as Random amplified polymorphic DNA (RAPD) analysis (Williams et al., 1990) has many advantages such as speed, low cost, minimal requirement of DNA, and lack of radioactivity, as a means of characterizing genetic variability. Major polymorphisms in RAPD pattern indicate genetic distinctness which can be used to distinguish unrelated groups. Minor polymorphisms may indicate genetic distinctness within groups or may occur because of experimental variability and, therefore, must be verified by repetition. RAPD analysis has been used effectively to distinguish between species of Fusarium (Mahdavi et al., 2009 and Mishra et al., 2010). Molecular techniques based on the polymerase chain reaction (PCR) have been used as a tool in genetic mapping, molecular taxonomy, evolutionary studies, and diagnosis of several fungal species (Nasir and Hoppe, 1991, Welsh et al., 1991 and McDonald, 1997).

The analysis of DNA products generated through RAPD has provided information on variation (Kerssies et al., 1997) and segregation of genetic traits among strains (Vander Vluget- Bergmans et al, 1993). RAPD analysis also offers several advantages that may be useful in studying formae speciales and races of Fusarium oxysporum (Pasquali et al., 2003, Lakhdar et al., 2004 and Bhim et al., 2006). Also, RAPD analysis has been used to characterize strains of many Fusarium spp. (Kini et al., 2002 and Liu Weicheng et al., 2002).

The earlier detection system involving isolation and purification of the bacterium from infected samples followed by biochemical analysis and pathogenicity tests is tedious and time-consuming (Nayar and Mathew, 1982; Franken and Vuurde, 1990).

Traditionally, nematode identification relies on light microscopy. A declining taxonomical skill base is problematical (Coomans, 2002) and compounded by time-consuming nature of the task. Several authors have proposed molecular methods as alternatives to morphological identification of soil nematode (Griffiths et al., 2006; Jones et al., 2006).

Coupled with this molecular method, an effective technique…... An effective and efficient detection should fulfill five criteria, i.e. quick, sensitive, accurate, applicable in the field, and economical (Seal and Elphinstone, 1994). Enzyme-Linked Immunosorbent Assay (ELISA) is a serological technique that meets these requirements. It is a very popular and promising technique for diagnosis of plant diseases.

In view of the above considerations, the present investigation was designed to detect the three vascular pathogens (fungal wilt, bacterial wilt and root knot) of tomato crop both in plant tissue and rhizosphere soils, with following objectives.

Objectives of Research

1. Molecular characterization (collected isolates) of different soil borne and vascular pathogens (Fusarium oxysporum f. sp lycopersici, Ralstonia solanacearum and Meloidogyne incognita) affecting tomato.
2. Development of antisera against these pathogens.
3. Molecular diagnosis of these pathogens

2. REVIEW OF LITERATURE

Tomato being an important vegetable crop is attacked by various plant pathogens (mainly, Fusarium oxysporum f. sp. lycopersici, Ralstonia solanacearum and Meloidogyne spp.). All these together or independently, are limiting factors in tomato production. The range of techniques available for identification and monitoring of soil-borne pathogens has expanded over past decades including immunological assays. Most recently, nucleic acid hybridization techniques are becoming very popular and promising techniques for diagnosis of soil borne diseases. Literature pertaining to the molecular and serological diagnosis of the forementioned vascular pathogens affecting tomato is reviewed here as under.

2.1 Report on occurrences of the forementioned pathogens

Fusarium wilt caused by the fungal pathogen Fusarium oxysporum Schlechtend. f. sp. lycopersici (Sacc.) W.C. Snyder & H.N. Hans. is known as one of the most devastating diseases of tomato (Solanum lycopersicum Mill.) worldwide (Walker, 1971, Jones et al., 1991).

Bacterial wilt caused by R. solanacearum is one of the most important diseases on agricultural crops, particularly on food and horticultural crops in tropical and subtropical regions of the world. Yield losses due to the disease caused by this bacterium on the different crops vary and are difficult to measure accurately, but are estimated at 15-35% on tomato (Hayward 1994; CAB International 2004). It affects a wide range of economically important crops such as tomato, potato, eggplant, chilli and non-solanaceous crops such as banana and groundnut. (Hayward, 1991; Grimault and Prior, 1994). In Taiwan, it has been reported that the incidence of bacterial wilt in tomato crops ranges from 15 to 55% during summer season. In India, a study showed 10 to 100% incidence of the disease during summer months. (Jaw-Fen Wang, 2005). Hartman (1991) reported that bacterial wilt of tomato caused by race 1 strain of R. solanacearum is a major disease in the tropics and subtropics. Disease incidences of 15 to 55% have been reported in fresh market tomato.

Similarly, root-knot nematodes belonging to the genus Meloidogyne are one of the most economically important plant pathogens (Siddiqi, 2000) especially in high value intensive crops. So far, over 95 species have been described, but more than 90% of the estimated damage worldwide is caused by four root-knot nematode species, namely M. incognita, M. javanica, M. arenaria, and M. hapla (Siddiqi, 2000; Netscher and Sikora, 1990).

2.2 Symptomatology

Symptoms of tomato wilt caused by F. o. f. sp. lycopersici have been described by Mui-Yun Wong (2003): the first indication of this disease is yellowing and drooping of the lower leaves. This symptom often occurs on one side of the plant or on one shoot. Successive leaves become yellow, wilt and die, often before the plant reaches maturity. As the disease progresses, growth is typically stunted, and little or no fruit develops. If the main stem is cut, dark brown streaks may be seen running lengthwise through the stem. This discolouration often extends far up the stem and is especially noticeable in a petiole scar. The browning of the vascular system is characteristic of the disease and generally can be used for its identification. Similar symptoms have also been reported by several workers from different ecological areas.(Bahattin, 2009; Davis, 1982; Beckman, 1987 and Duniway, 1971)

Janse et al. (2004) reported that bacterial wilt caused by R. solanacearum in tomato plants were characterized by wilting of upper leaves during the hottest part of the day followed by recovery during the evening and early hours of morning. Under favorable condition complete wilt occured. The vascular tissues in the lower stem of wilted plants show a dark brown discolouration. These symptoms are similar to those of some fungal diseases. A cross section of stem of the plant with bacterial wilt produces a white, milky stand of bacterial cell in clear water. This ooze distinguishes the wilt caused by the bacterium from that caused by fungal pathogen. James et al. (1993) observed R. solanacearum producing water soaked, glistening golden brown patches on the leaves with irregular margin.

Brown discolouration of root and stem tissue with decaying of roots and sometimes shredding of roots was seen. James et al. (1994) reported the bacterial wilt of tomato was characterized by yellowing of the leaves followed by wilting and defoliation. The entire plant dried up within two to three weeks time.

Nickle (1991) observed tomato plants infested with root knot nematodes were stunted in their growth with yellow coloured leaves. When infected plants were uprooted, deformed roots with prominent multiple galls of varying size were noticed. Microscopic examination of the galls revealed the presence of egg, juveniles and females of M. incognita in the vascular region of roots. The presence of egg masses outside the gall was a common phenomenon noticed in tomato plant. Their parasitic life style in the roots of plants results in root hyperplasia and galling which debilitates the root system (Taylor, 1971).

2.2.1 Morphological and cultural characters and pathogenicity of Fusarium oxysporum f. sp. lycopersici

A major problem in working with the vascular wilt isolates of F. oxysporum is the maintenance of the original morphology and virulence of the organism. Starting from the sporodochial type, variation in general, proceeds either towards type producing abundant aerial mycelium but few macroconidia (termed mycelial types) or towords types producing little mycium or no aerial mycelium but abundant macroconidia (termed pionnotal types) Nelson (1981). Booth (1971) the morphology of conidiophore was used as a primary basis for species identification. White (1972) has grouped 24 strains into two groups based on morphological, cultural and pathogenicity. He observed differences in pigmentation, mycelial charecters, sporulation and the isolates differed markedly in inducing wilt. Similarly the grouping of Fusarium spp. on the basis of morphological, cultural and pathogenic characters were used by other workers Varma (1954), Goswami and Baruah (1956).

2.3 Molecular variability

2.3.1 Random amplified polymorphic DNA (RAPD) analysis for Fusarium oxysporum f. sp. lycopersici

The information about moleculer polymorphism is reviewed here with special emphasis on Random amplified polymorphic DNA (RAPD).

The RAPD technique has quickly gained widespread acceptance and application because it is a relatively simple genetic tool to experiment in biological systems. However, RAPD is the best assay when the nucleotide sequence is not known. Unlike other PCR protocols which utilize two primers of defined sequence, RAPD detects nucleotide polymorphism using only one primer of an arbitrary nucleotide sequence. Random amplified polymorphic DNA (RAPD) analysis has many advantages as a means of characterizing genetic variability such as speed, low cost, minimal requirement for DNA, and lack of radioactivity (Williams et al., 1990).

Polymerase chain reaction (PCR) has been widely and successfully employed for the diversity analysis of the important plant pathogenic fungi including Fusarium sp. Kim et al. (1993), Kini et al. (2002) and Liu Weicheng et al. (2002). The analysis of DNA products generated through random amplified polymorphic DNA has provided information on variation (Kerssies et al., 1997) and segregation of genetic traits among strains (Vander Vluget-Bergmans et al., 1993). Thirty isolates of Fusarium oxysporum f. sp. ciceri were isolated from rhizosphere soil of chickpea from different locations in northern India. The amount of genetic variation was evaluated by RAPD with a set of 40 primers. Less than 10% of the amplified fragments in each case were polymorphic. Genetic similarity between each of the isolates was calculated and results indicate that there was little genetic variability among the isolates collected from the different locations Bhim et al. (2006). Gopal et al. (2004) Random amplified polymorphic DNA was used to identify genetic variation among 22 isolates of F. o. f. sp. fragariae. All isolates could be distinguished from each other by RAPD analysis. Based on the banding patterns results indicate that F. o. f. sp. fragariae isolates are genetically distinct and there was a high level genetic variation from each other.

El-Fadly et al. (2008) RAPD-PCR, was used to identify. Seven Fusarium isolates which were identified by their morphological and pathological characteristics as F. semitectum, F. culmorum, F. moniliforme, F. solani, F. raminearum, F. oxysporum f. sp. lycopersici and F. oxysporum f. sp. vasinfectum. Six out of the eight primers differentiated between some of the tested Fusarium species, since 100% similarity was recorded between two or three different species of the fungus. While, the rest two primers clearly distinguished each of all studied Fusarium spp. including the two formae speciales. In conclusion, RAPD-PCR technique is a useful tool for differentiating between species and formae speciales of the genus Fusarium either alternatively or complementary to methods based upon morphological and pathological characteristic. Manulis et al. (1994), Pasquali et al. (2003) and Lakhdar et al. (2004).

Mishra et al. (2010) studied inherent diversity among F. o. f. sp. lycopersici isolates, from different tomato growing regions in India, determined using RAPD primers. The genetic similarity coefficients ranged from 0.20 to 0.96, indicating that no any two or more isolates were 100% similar. RAPD profiles revealed up to 20% genetic diversity among ten isolates of F. o. f. sp. lycopersici. Mahdavi et al. (2009) seventy isolates of F. oxysporum were isolated from infected tomato plants. Cluster analysis of RAPD data showed, 8 main groups at 71% similarity level. Also, 18 molecular phenotypes were detected among isolates. The low number of distinguished groups observed among the populations could suggest that these isolates have probably originated from a common ancestor and sexual reproduction has played a minor role in genetic variability of the fungus in its life cycle.

Some studies have indicated that RAPD patterns can be used to distinguish isolates with differing levels of virulence. Honnareddy and Dubey (2006) the isolates of F. o. f. sp. ciceris were collected from different part of India. Pathogenic virulence study of 25 isolates of the pathogen on international set of differential cultivars revealed the existence of three new races of the pathogen in India which was assessed by RAPD with a set of 40 ten -mer primers. Assigbetse et al. (1994) RAPD markers were used to assess genetic diversity among 46 isolates of F. o. f. sp. vasinfectum of worldwide origin. And isolates are clustered into three groups corresponding to their pathological reactions.

Sharma et al. (2006) studied genetic diversity in F. o. f. spp. pisi isolates from three agronomically distinct regions. Sub-tropical, sub-humid and wet-temperature of north-western Himalayas, was studied based on NTSYS analysis of RAPD data. The isolates were delinated into four region specific groups, group PRI, PRIII and PRIV represented isolates from sub tropical and sub humid regions whereas group PRII from dry temperate region.

2.3.2 Random amplified polymorphic DNA (RAPD) for Ralstonia solanacearum

Deepa et al. (2003) used nine strains of R. solanacearum isolated from bacterial wilt affected plants in three agro-climatic zones of Kerala. Isolates grouped into Biovar III belonged to Race 1 and 3 and OPF 8 primer yielded a unique band of 1.43 kb size for race 3. This was considered as a marker for detection of race 3. Jaunet and Wang (1997) studied the genetic diversity among the biovars of the pathogen. DNA amplification with different primers revealed great diversity in the population of R. solanacearum Biovars III and IIIA. None of the primers yielded bands specific to any of the two biovars.

Gunathilake et al. (2004) reported that genetic diversity of R. solanacearum which was assessed using RAPD method with ten selected decamer primers, namely OPA-02, OPA03, OPA-04, OPA-14, OPC-15, OPD-07, OPD-08, OPD-10, OPD-15 and OPF-12. A higher degree of polymorphism was observed even in the isolates collected from same agroecological zone. RAPD data indicated that a higher genetic diversity of pathogen in three districts of Kandy, Matale and Monaragala. No clear relationship between relative pathogencity value was observed and clustering. Later, Grover et al., (2006) A total of 44 field isolates and 22 in vitro generated clones of R. solanacearum were studied for genotypic diversity by random amplified polymorphic DNA (RAPD) technique with 30 decamer primers. The data were analysed using NTSYSpc 2.02h software. Forty-two out of 44 field isolates and all the clonal isolates were identified as distinct genotypes at 70% similarity level.

2.3.3 18 s - rDNA analysis of Meloidogyne incognita

Relatively highly conserved sequences of internal transcribed spacer (ITS) regions of rDNA encoding the structure of ribosomes are often used for detection purposes (Zijlstra et al., 1997 and Williamson et al., 1997).The 18S rDNA gene has been used extensively in nematode phylogeny (Blaxter et al., 1998).

Significance of rRNA in phylogenetic inference: As with other eukaryotes, the nematode rDNA cistron typically consists of several hundred tandemly repeated copies of the transcribed units (small subunit or SSU or 18S; large subunit or LSU or 28S; 5.8S; internal and external transcribed spacers) and an external nontranscribed or intergenic spacer (Hillis and Dixon, 1991).

18s- rDNA of 19 populations of Meloidogyne spp. were amplified and directly sequenced by Tigano et al. (2005) for 18S rDNA data, three main clades were identified. One well supported clade (86-91% bootstrap) included the most common and widely disseminated species, e.g. M. arenaria, M. javanica and M. incognita, some recently described or redescribed species (M. floridensis, M. paranaensis, and M. ethiopica) plus numerous unidentified isolates. The nearly 18s rDNA sequences obtained from the 19 taxa varied from 1111 bp (for M. floridensis) to 1714 bp (for M. incognita). Cliff and Hirschmann (1985) and Rammah and Hirschmann (1990) reported isolates of M. arenaria and M. morocciensis produced amplified 18s fragment of the same size (1112bp).

2.4 Serodiagnosis

2.4.1 Serological detection of Fusarium oxysporum f. sp. lycopersici

Coons and Strong (1928) were the first to employ serological techniques in identifying the genus Fusarium. Since then, few attempts have been made to utilize this technique for study of Fusarium. Sayed et al. (1976) attempted Immunoserological investigation of three formae speciales of F. oxysporum, viz. f. sp. ciceri, udum and vasinfectum revealed a close relationship between F. oxysporum f. sp. udam and F. oxysporum f. sp. vasinfectum. Madhosingh (1964) compared F. moniliformae, F. oxysporum and F. solani serologically.

The basic technique of ELISA is serology, the reaction between antigen (Ag) and antibody (Ab). This technique, however, needs certain enzymes and substrates to label the reaction that enable to produce colour that readable by naked eyes or using an electronic tool, ELISA Reader (Converse and Martin, 1990; McLaughlin and Chen 1990). The original ELISA technique has been modified to improve its effectiveness and given different names, such as Double Antibody Sandwiched-ELISA (DAS-ELISA), Direct ELISA, Indirect ELISA, and Dot Blot ELISA (Stobbs and Barker 1985, Yadi et al., 1998).

Soo Bong Park et al. (2004) developed a polyclonal antibody based-ELISA system to monitor inocula accurately and rapidly before onset of anthracnose on soybean sprouts. Titer of antisera against conidia of Colletotrichum gloeosporioides, determined by indirect ELISA, was high enough to be detectable up to x25,600 dilutions. Jung Han Lee et al. (2004) reported that polyclonal antibody raised against C. gloeosporioides was precise enough to detect spore concentrations of as low as 500 conidia/well by Indirect –ELISA technique.

Mahadevan (1964) attempted to determine antigenic variation between the spore and mycelium of F.o. f. sp. lycopersici employing serology., He concluded that the antigenic composition of mycelium and spore appeared to be relatively same, although spores do have more antigenic properties.

Mahmoud et al. (2010) studied the reactivity of Phytophthora infestans antiserum to the purified antigen of P. infestans using Indirect- ELISA. The antigen was diluted with phosphate buffer to adjust the given concentration (0.1- 50 µg). Data showed that, the antigens at concentrations of 5-50 µg were detected using Indirect- ELISA (positive ELISA values). In contrast, antigens of 1 and 0.1 µg were not detected (negative ELISA values). Gautam et al. (1999) reported the lack of specificity in the antiserum with Pythium sp. may be attributed to the very close taxonomical relation between Pythium sp. and P. infestans. This indicated that there are also many molecular epitopes on the fungal surface, which are common to a Phytophthora and Pythium species.

Polyclonal antisera were prepared against purified mycelial proteins from Veticillium dahliae. These antisera tested by using ELISA against Verticillium sp, showed a positive result. The antisera did not react with Fusarium and Colletotrichum sp. (Sundarum,1991).

Ivana et al.(2002) raised antisera against Rhizoctonia which showed a positive reaction with samples of strawberry roots infected with binucleate Rhizoctonia The reaction was absent with antigens from other fungal genera (Agaricus sp., Pythium sp. and Fusarium sp.), and with healthy root samples .

2.4.2 Serological detection of Ralstonia solanacearum

In recent years research has been directed towards developing rapid, sensitive and specific diagnostic assays to detect the presence of R. solanacearum in plant and soil samples (Baker et al., 1984 and Hendrick and Sequiera, 1984). The enzyme-linked immunosorbent assays (ELISA) for the detection of R. solanacearum developed and employed to detect R. solanacearum in infected potato (Stackebrandt et al., 1988; Robinson, 1992; Robinson et al., 1994 and Anonymous, 1997) and different solanaceous plant materials using polyclonal (PAb) and monoclonal antibodies (MAb).

The agglutination test was used to distinguish different species or strain of Xanthomonas spp. (Fang et al., 1950). The eight species studied were differentiated into five serogroups based on serological relationship (Patel et al., 1951). Xanthomonas translucens that causes bacterial stripe blight in cereal and grasses was differentiated into five special forms (strains) by Fang et al. (1950). Lyons and Tylor (1990) developed a rapid slide agglutination test that uses polyclonal antisera conjugated to Staphylococcus aureus cells which high concentrations of protein a on their surface. This test could used for the detection of Pseudomonas syringae pv. phaseolicola and Pseudomonas syringae pv. pisi in lesion on bean and pea, respectively. P. gladioli pv. alllicola and Lactobacillus sp. Were detected in rotted onion bulbs. The presence of specific strains of Rhizobium phaseoli in bean rot nodules could also be detected.

Machmud and Yadi (2008) reported that results of the three ELISA techniques, i.e. Direct-ELISA, Indirect-ELISA and NCM-ELISA showed different effectiveness of detection. The Direct -ELISA technique was capable of detecting R. solanacearum of 10^3 cells/ml, while the Indirect- ELISA and NCM -ELISA technique detected only up to 10^4 cell/ml. Rajeshwari et al. (1998) developed a serological assay to screen the infected plant materials and seeds of tomato affected by R. solanacearum. Their results indicated that the assay was vary specific and reactive to all isolates R. solanacearum from tomato. Had a good antibody titre of 1:10,000 and could detect the bacterium at all numbers as low as 100 cell per ml.

2.4.3 Serological detection of Meloidogyne incognita

Bird (1964) was the first to report the possibility of generating antibodies against nematodes.

Alan (1964) reported the injection of living larvae of the plant parasitic root-knot nematode, M. javanica into rabbits results in the production of antibody. Antigenic material exuded from the excretory pore and to a lesser extent, from the buccal stylet of both larvae and adults. The gelatinous matrix exuded from the adult female also appeared to have antigenic properties as, to a lesser extent, did the cuticle. As the larvae aged, fewer produced precipitates in antiserum. Celeste et al. (1996) Polyclonal antiserum was raised against the major 76 kDa collagen protein extracted from cuticles of M. incognita adult females.

Liziane et al. (2005) tested polyclonal antibodies with antigens present in the homogenates of J2 of M. incognita and M. arenaria using Indirect ELISA assay. Evans (1997) Monoclonal antibodies (MAbs) and polyclonal antibodies were produced to antigen of an Australian population of cereal cyst nematode (CCN). Monoclonal antibodies IACR, ccnj-49.2 (molecular weight 200 kDa) recognized an antigen, which was immunolocalized apparently in granules in the nematode gut. By using ELISA format, same (the MAbs) was used to identify CCN population from soil containing 20 per cent organic matter. Davis (1995) reported that the polyclonal antibodies have been used for quantification of Meloidogyne spp. Results showed that polyclonal antibodies were highly sensitive and could sufficiently quantify small number of nematode population per g soil.

3. MATERIAL AND METHODS

Present investigations were carried out during 2007 to 2010. Laboratory experiments were carried out at the Department of Plant Pathology and Institute of Agri -Biotechnology (IABT), College of Agriculture, University of Agricultural Sciences, Dharwad, The materials used and the techniques adopted during the course of investigations are described in this chapter.

General laboratory procedure

Glassware cleaning

For all the laboratory experimental studies, Corning and Borosil glassware were used. The glassware were kept in cleaning solution for 24 h, containing 60 g of potassium dichromate ($K_2Cr_2O_7$), 60 ml of concentrated sulphuric acid (H_2SO_4) in 1000 ml of water. They were washed with vim powder followed by washing in running tap water and then rinsed in distilled water before use.

Sterilization

All the glassware were sterilized in an autoclave at 1.1 kg/cm^2 pressure for 20 minutes. All the media were sterilized for 15 minutes at 1.1 kg/cm^2 pressure and soil used for experiment was sterilized at 1.33 kg/cm^2 pressure for two hours.

Media

Name of the media used, their composition and method of preparation are briefly described hereunder.

a) Potato dextrose agar (Tuite, 1969)

1. Peeled and sliced potatoes : 200 g
2. Dextrose ($C_6H_{12}O_6$) : 20 g
3. Agar agar : 20 g
4. Distilled water (to make up) : 1000 ml

The peeled and sliced potatoes were boiled in 400 ml of distilled water and the extract was collected by filtering through a muslin cloth. Agar-agar was melted separately in 400 ml of distilled water. The potato extract was mixed in the molten agar and 20 g of dextrose was added to the mixture. The volume was made up to 1000 ml with distilled water and sterilized at 1.1 kg/cm^2 pressure for 15 min.

b) Sucrose peptone agar

Sucrose ($C_{12}H_{22}O_{11}$)	: 20g
Potassium dihydrogen phosphate (KH_2PO_4)	: 1g
Magnisium chloride ($MgCl_{2)}$	: 5g
Peptone	: 5g
Agar	: 15-18g
Distilled water	: 1000 ml
pH	: 7.2

Just before pouring into the plates. 500 µ1 of stock solution of sterilized 2, 3, 5-

Triphenyl tetrazolium chloride (stock 10mg/ml) was added.

3.1 Collection of samples

Wilt affected tomato plants were collected from different parts of Karnataka. Fusarium affected samples were collected from 23 locations. Where as Ralstonia affected samples were collected from 24 locations. The details of location and designation given for each isolates are furnished in Table1 and Table 2.

Table 1. Fusarium oxysporum f. sp. lycopersici isolates from major tomato growing areas of Karnataka

District	Location	Isolate number
Dharwad	Saidapur	Fol-1
	Garag	Fol-2
Bangalore	Nelamangala	Fol-3
	Doddaballapur	Fol-4
Tumkur	Gubbi	Fol-5
	Hosalli	Fol-6
Gadag	Bannikoppa	Fol-7
	Lakkundi	Fol-8
Haveri	Hangal	Fol-9
	Ranebennur	Fol-10
Belgaum	Arabhavi	Fol-11
	Khanapur	Fol-12
	Gokak	Fol-13
Mysore	Mysore	Fol-14
	Hunsur	Fol-15
Chikkballapur	Chikkballapur	Fol-16
	Chintamani	Fol-17
Shimoga	Shimoga	Fol-18
	Sagar	Fol-19
Ramanagar	Ramanagar	Fol-20
Kolar	Kolar	Fol-21
Chikkmanglur	Chikkmangalur	Fol-22
Davanagere	Davanagere	Fol-23

Table 2. Ralstonia solanacearum isolates from major tomato growing areas of Karnataka

District	Location	Isolate number
Dharwad	UAS Dharwad	Rs-1
	Garag	Rs-2
	Sapthapur	Rs-3
Bangalore	UAS Bangalore	Rs-4
	Doddaballapur	Rs-5
Tumkur	Gubbi	Rs-6
	Hosalli	Rs-7
Gadag	Bannikoppa	Rs-8
	Lakkundi	Rs-9
Haveri	Ranebennur	Rs-10
	Chalageri	Rs-11
Belgaum	Arabhavi	Rs-12
	Gokak	Rs-13
	Kanapur	Rs-14
Mysore	Mysore	Rs-15
	Hunsur	Rs-16
Chikkballapur	Chikkballapur	Rs-17
	Chintamani	Rs-18
Shimoga	Shimoga	Rs-19
	Sagar	Rs-20
Ramanagar	Ramanagar	Rs-21
Kolar	Kolar	Rs-22
Chikkmanglur	Chikkmangalur	Rs-23
Davanagere	Davanagere	Rs-24

3.2 Isolation and maintenance of Fusarium oxysporum f. sp. lycopersici

Tomato plants showing typical symptoms of Fusarium wilt were collected from different major tomato growing areas of Karnataka. The infected seedlings showing typical symptoms of the disease were used for isolation of pathogen. The standard tissue isolation procedure was followed for isolation of pathogen. The infected parts were surface sterilized with 1 per cent sodium hypochlorite solution for 60 seconds and washed serially in distilled water to remove the traces of sodium hypochlorite and then transferred to sterilized petriplate containing potato dextrose agar (PDA). The petriplates were incubated at room temperature (27±1 °C) and observed periodically for the growth of colonies. The colonies which developed from the bits were transferred to PDA slants and incubated at 27 ± 1°C. Such slants were used to study characters.

3.2.1 Hyphal tip isolation

This method was followed for maintaining pure culture, since Fusarium oxysporum is known to be heterokaryotic in nature. Hyphal tip isolation was done on water agar plates. Dilute spore suspension (8-10 spores/ml) was prepared in sterile distilled water. One ml of such suspension was spread uniformly on two per cent water agar plates and the excess of which was aseptically drained. Single spore was then marked under the microscopic field with ink on the glass surface of the plate and it was allowed to germinate. Such plates were incubated at 27±1 °C and periodically observed for germination of spores under the microscope. Hyphae coming from each end cell of the single spore was traced and marked with the ink. Then tip of hypha was cut and transferred to PDA slants with the help of cork borer under aseptic conditions and incubated at temperature of 27±1 °C. Later, mycelial bits of the fungus were placed in the center of petriplates containing potato dextrose agar medium and incubated at 27±1 °C. No saltation or sectoring was observed in the culture and it was concluded that it was a pure culture of the fungus. Such culture was used for further studies.

3.2.3 Maintenance of the culture

The fungal pathogen was sub cultured on PDA slants and allowed to grow at 27±1 °C and such slants were preserved in refrigerator at 5 °C and revived once in 30 days.

3.2.4 Morphological and cultural character

Twenty three isolates of Fusarium spp. obtained upon isolation from wilted tomato plants were compared for variation in morphological and cultural characters on PDA medium. Twenty ml of medium was poured into each sterilized petriplate and five mm mycelial disc from actively growing seven days-old culture of each isolates of Fusarium sp. was inoculated at the centre of PDA and petri plates were incubated at 27±1 °C for seven days. Observations on colony colour and linear growth measurements were recorded upto seven days. Spore measurements were taken with the help of filar micrometer.

3.2.5 Pathogenicity

The fungus was multiflied on PDA under aseptic conditions in petriplates. When fully covered with fungal growth (seven days old) the growth was harvested with the help of a sterile scalpel and washed with 100 ml of sterile water. The contents of each plate were filtered through a muslin cloth to get a spore suspension. The suspension was adjusted to have a concentration of $5x10^6$ spores/ml of sterile water. Four holes were made in the soil around collar region of each seedling four weeks grown in pot containing sterilized soil. The holes, 4-5 cm deep with the help of a small sticks. Ten ml of the spore suspension was poured into the holes and covered with soil. Control plants were applied with sterilized tap water (Kesavan and Chowdhury, 1977).

Disease severity index was evaluated by using following scale (Bora et al., 2004)

0 = No symptoms
1 = <25% leaves with symptoms
2 = 26-50% leaves with symptoms
3 = 51-75% leaves with symptoms
4 = 76-100 leaves with symptoms

3.3 Isolation of Ralstonia solanacearum from tomato plants

Tomato plants showing typical symptom of wilting caused by R. solanacearum were collected. Tentative diagnosis of the disease was done by ooze test. The bacterium was isolated by extracting the ooze in sterile distilled water taken in test tube, followed by dilution plate technique on TZC medium. Small pieces of infected vascular tissue were aseptically cut with a sterile surgical blade after removing the bark. The infected tissue bits were surface sterilized in 70 percent alcohol for a minute and were washed in two to three series of sterile water to remove traces of alcohol. The infected tissues were then suspended in a sterile water taken in test tube which became turbid due to oozing of bacterial cells from the infected tissue The bacterial suspension was serially diluted and 100 µl of the diluted bacterial suspension was poured onto the surface of solidified Triphenyl tetrazolium chloride (TZC) medium with a sterilized spreader. The inoculated plates were incubated at 32^{o}C for 48 h. The plates were observed for the development of well separated virulent colonies.

3.3.1 Purification and preservation of Ralstonia solanacearum

Well separated virulent colonies of R. solanacearum on TZC medium were picked up and streaked on the surface of TZC medium. The virulent colonies of R. solanacearum were irregular, dull white, fluidal, slimy with slight pink center. For the purpose of purification, the well separated typical virulent colony was picked up and streaked on the surface of TZC medium and plates were incubated at 32 ^{o}C for 48 hours. Three to four loopful of the bacterial culture were picked up from the well separated colonies from streaked TZC medium and suspended into 2 ml sterilized distilled water contained in small polyprolene culture tubes. The inoculated tubes were kept at room temperature which served as stock culture for further use.

3.3.2 Pathogenicity

Inocula for pathogenicity tests were prepared with 48 h old cultures grown on TZC medium at 32 ^{o}C. Colonies were suspended in sterilized water to get $1x10^{-8}$ cfu /ml using a spectrophotometer, transmission at A600=0.1 and were used for inoculation immediately after preparation. The soil mixtures used in the research were steam sterilized for 2 h. Fourteen days old seedlings of Pusa ruby (Susceptible check variety) were grown in the pot containing sterilized soil mixture as one plant per pot and maintained under green house conditions. Root inoculation was done at, prepferably third true leaf stage or slightly older Root zone was damaged using a sterilized scalpel and 50 ml of inoculum culture was introduced to each plant. Control plant were applied with sterilized tap water (Somodi et al., 1992).

Disease severity was recorded on a 1-5 scale:
1= no visible symptoms
2=1-25 % of the plant is wilting
3=26-50% wilt
4=51-75% wilt
5= more than 75% wilts

And virulence of each strain was rated as low virulence =1.1-2.5; medium virulence =2.6-4.0 and high virulence = 4.1- 5.0 (Kelman and Person, 1961).

3.4 Collection, maintenance and identification of root-knot nematodes

3.4.1 Collection and maintenance of root knot nematode

Root knot samples were collected from Dharwad (UAS Dharwad and Saidapur), Belgaum (Arabhavi), Bangalore (UAS Bangalore).

Root-knot infected tomato plants were collected in polythene bags and kept in the freezer. Root portion was carefully removed from the soil and washed gently under running tap water. Egg masses were picked and kept for hatching in water in a petridish. After 24-36 hours, hatched juveniles were used to inoculate, tomato plants grown in sterilized soil and sand (1:1) mixture in greenhouse. These plants served as culture plants. After giving sufficient time so as to complete 2-3 generations of the nematode, the plants were depotted carefully. The root systems were washed free of soil, the galls containing egg masses were used to get inoculum of the nematode for further studies throughout.

3.4.2 Identification of Root knot nematode species

The galled root system was immersed in boiling 0.1 per cent cotton blue in lactophenol and left overnight for clearing (Southey, 1986). The roots infected by root knot nematode were washed. The females were dissected out from the well developed galls of the roots under the stereobinocular microscope and were transferred to a drop of lactophenol taken on a clean glass slide. The posterior portion of the females was cleaned. The perineal region was trimmed and mounted for observation under oil immersion objective of a compound microscope. The identification of the species was made on the basis of characters of perineal pattern described by Eisenback et al. (1981).

3.4.3 Proving pathogencity of root-knot nematodes

Pathogencity of all collected isolates were proved by inoculating nematode suspension as mentioned above, on fourteen days old tomato seedlings were grown in the pot containing sterilized soil mixture as one plant per pot and maintained under green house condition. A similar treatment was given to the uninoculated check plant except that only water was used instead of the nematode suspension. After giving sufficient time (60-100 days) so as to complete 2-3 generations of the nematode, the plants were depotted carefully. The root systems were washed free of soil, and were examined for presence of gall (Mc Beth et al., 1941).

3.5 Molecular diagnosis

3.5.1 Molecular variability of Fusarium oxysporum f. sp. lycopersici

For variability studies, twenty three isolates of F. o f. sp lycopersici were used collected from different places as mentioned already.

Random amplified polymorphic DNA (RAPD) analysis was used to detect the variation among the isolates of F. o. f. sp lycopersici. The lyophilized cultures were used for genomic DNA isolation according to the method of Rehner and Samuels (1994) with minor modifications.

3.5.1.1 Requirements

Stock solutions

2% SDS : Two grams of sodium dodyecyl sulphate was dissolved in 100 ml of distilled water

Lysis buffer : 2.5 mM EDTA
1% Triton X
50 mM Tris HCL
pH 8.0

Phenol: Chloroform : Isoamyl alcohol-25:24:1

T_{10} E_1 : 10 mM Tris
1 mM EDTA
pH 8.0

100µM of random primer
25 ng /µl template DNA
3.0 U/µl Taq DNA polymerase

3.5.1.2 Isolation of template DNA

I. Procedure

- 2-3 g of seven day old fungal mat grown on potato dextrose broth was taken and homogenized using pestle and mortar in 4 ml of 2 per cent SDS for 5 minutes.

- Six ml of lysis buffer was added to the above solution
- The suspension in pestle and mortar was extracted with equal volume of Phenol: Chloroform: Isoamyl alcohol (1:1 V/V) in centrifugation tube, centrifuged at 10000 rpm for 10 minutes.
- Supernatant was taken in fresh centrifuge tube. To this, $1/10^{th}$ volume of 3M sodium acetate and 0.54 volume of isopropanal were added. Mixed by gentle inversion and kept for 30 minutes at –20 ^{o}C.
- The DNA was recovered by centrifugation at 10000 rpm for 10 minutes at 4 ^{0}C. The DNA pellet was washed with 70 per cent ethanol, air dried and re-suspended in 300µ1 of $T_{10}E_{1.}$ This DNA obtained was further quantified by agarose gel electrophoresis.

II. Random primers

Commercial kit OPA, OPB and OPF of decamer DNA primers were obtained from M/s Bangalore Genei, Pvt. Ltd. Bangalore.

III. dNTPS

The four dNTPs such as dATP, dGTP, dCTP and dTTP were obtained from M/s Bangalore Genei, Pvt. Ltd. Bangalore.

IV. Taq DNA Polymerase

The Taq DNA Polymerase with 10xTaq buffer was abtained from M/s Bangalore Genei, Pvt. Ltd. Bangalore.

V. Thermo cycler

Eppendorf Master cycler gradient supplied by Eppendorf Gradient, 2231, Hamburg Germany was used for cyclic amplification of DNA.

3.5.1.3 The thermoprofile for PCR

The PCR amplification for RAPD analysis was performed according to Williams et al. (1990) with certain modifications. The optimum condition for DNA amplifications used were as follows:

Step	Temperature (^{o}C)	Duration (min)	Number of cycles
Denaturation	94	4	1
Denaturation	94	1	40
Annealing	36	1	
Extension	72	2	
Final extension	72	5	1
Hold temperature	4	-	-

After the completion of the PCR, the products were stored at 4 ^{o}C until the gel electrophoresis was done.

A total of 30 random primers with the following sequences belonging to operon A,B and F series was used in the study.

3.5.1.4 Master mix for PCR

Reaction mixture was prepared in 0.2 ml thin walled PCR tubes containing the following components. The total volume of each reaction mixture was 20 µl. The following reaction mixture was found to be optimum for PCR amplification.

10x assay buffer with 15 mM $MgCl_2$: 2.5 µl
dNTPs mix (2.5 mM each) :1.0 µl
Primer (5pM/ µl) : 1.0 µl
Template DNA (25 ng/ µl) : 1.0 µl
Sterile distilled water : 14.30 µl
Taq DNA polymerase (3.0U/ µl) :0.2 µl

3.5.1.5 Separation of amplified products by agarose gel electrophoresis

Requirement

Electrophoretic unit: gel casting tray, gel combs, power-pack, UV-transilluminator
Agarose (1.5 %)
Bromophenol blue
Ethidium bromide (loading dye)

1X TAE buffer: 20ml 50X TAE was added to the 980 ml of distilled water and total volume was made upto 1000ml.

Sequences of random primers used in RAPD Analysis

Primer	Sequence
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCTG
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCYGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPB-01	GTTTCGCTCC
OPB-02	TGATCCCTGG
OPB-03	CATCCCCCTG
OPB-04	GGACTGGAGT
OPB-05	TGCGCCCTTC
OPB-06	TGCTCTGCCC
OPB-07	GGTGACGCAG
OPB-08	GTCCACACGG
OPB-09	TGGGGGACTC
OPB-10	CTGCTGGGAC
OPF-01	ACGGATCCTG
OPF-02	GAGGATCCCT
OPF-03	CCTGATCACC
OPF-04	GGTGATCAGG
OPF-05	CCGAATTCCC
OPF-06	GGGAATTCGG
OPF-07	CCGATATCCC
OPF-08	GGGATATCGG
OPF-09	CCAAGCTTCC
OPF-10	GGAAGCTTGG

Composition of 50X TAE buffer

Tris base	: 240.0g
Glacial acetic acid	: 57.1 ml
EDTA (0.5 M, pH 8.0)	: 100 ml

Volume was made upto 1000 ml using distilled water.

Procedure

- Three grams of agarose was weighed and added to a conical flask containing 250 ml of 1X TAE buffer.
- The contents were melted by heating in an oven, the solution was stirred for proper mixing and complete dissolution of agarose.
- The agarose gel solution was cooled to about 40 to 45 ^{o}C and 2-3 drops of ethidium bromide (0.5µg/ ml) was added.
- Agarose gel was poured into the casting platform after inserting the comb in tray. While pouring, sufficient care was taken to prevent the formation of air bubbles.
- The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoretic apparatus containing sufficient buffer (1X TAE), So as to cover the wells completely.
- About 2.5 µl of loading dye was added to each tube containing amplified DNA.
- Amplified products (20 µl) were carefully loaded into the sample wells.Electrophoresis was carried out at 60 volts until the tracking dye migrated to the end of the gel.Gel was viewed under UV transilluminator for DNA bands and photographed for documentation.

3.5.1.6 Scoring of amplified fragments

The amplified profiles for all primers were compared with each other and bands of DNA fragment were scored as 1 for presence and 0 for absence, generating 0 and 1 matrix. Per cent polymorphism was calculated by using the formula.

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

3.5.1.7 Analysis of the profile of the amplified fragments

Pair-wise genetic similarities between isolates were estimated by DICE similarity coefficient. Clustering was done using the symmetric matrix of similarity coefficient and cluster obtained based on unweighted pair group arithmetic mean (UPGMA) using sequential agglomerative hierarchal nested (SAHN) cluster analysis of NTSYS-PC version 2.0 (Rohlf,1998).

3.5.2 Molecular variability of Ralstonia solanacearum

Twenty four isolates of R. solanacearum collected from different tomato growing regions of Karnataka were used in this study.

3.5.2.1 Requirements

Stock solutions

50 M Tris HCL
100 mM NaCl
$T_{50}E_{20}$
Lysozyme (50mg/ml)
RNase (10mg/ml)
SDS (2% in $T_{50}E_{20}$)
Phenol:Chloroform:IAA (25:24:1)
3M Sodium acetate

3.5.2.2 Total genomic DNA isolation

Total genomic DNA isolation was performed as described by Sambrook and Rausell (2001).

- Pure culture of each isolate was streaked into the triphenyl tetrazolium chloride (TZC) medium and incubated at 32 ^{o}C for 48 h.
- Single colony of each isolate grown on triphenyl tetrazolium chloride (TZC) medium was inoculated to 25 ml of nutrient broth taken in 100 ml flasks. The flasks were kept for incubation at 32 ^{o}C for 24 h.
- Aliquots of culture were centrifuged at 10000 rpm for 10 min at 4 ^{o}C.
- The supernatant was poured off, 1.5 ml 10mM Tris-Cl and 1.5 ml of 100 mM NaCl was added and mixed well, contents were centifuged at 10000 rpm for 10 min at 4 ^{o}C.
- The pellet was resuspended in 2.5 ml of $T_{50}E_{20}$, 0.5 ml of lysozyme (50mg/ml) was added and incubated at 37 ^{o}C for 20 min, to which 25 µl of RNase (10mg/ml) was added, mixed well and incubated at room temperature for 10 minutes.
- 2.5 ml of SDS (2% in $T_{50}E_{20}$) was added, mixed by gentle inversion incubated at 50 ^{o}C for 45 min,
- The contents were treated with 50 µl of proteinase K (20mg/ml) incubated at 50 ^{o}C for 10 min.
- An equal volume of phenol was added mixed gently by inverting the tubes, centifuged at 10000 rpm for 10 min at 4 ^{o}C.
- The upper aqueous phase was transferred to fresh tube, An equal volume of Phenol: Chloroform: IAA (25:24:1) was added and mixed gently, centifuged and separated aqueous phage.
- An equal volume Chloroform: IAA was added centifuged and separated aqueous phage.
- 1/10th volume of 3M Sodium acetate was added, to this equal volume of isoamyl alcohol was added, the tubes were kept in ice for 20 min. The DNA was recovered by centrifuged at 10000 rpm for 10 min at 4 ^{o}C.
- The DNA pellet was washed with 70 per cent ethanol, centrifuged 2 min and supernatant was decanted, air dried and re-suspended in 25-30 µ1 of $T_{10}E_{1.}$

3.5.2.3 DNA quantity and quality estimation

To test the quality, DNA samples were run on 0.8 per cent agarose gel in 1X TAE buffer and stained with ethidium bromide and checked for contamination by RNA (which usually runs ahead) and the DNA was evaluated by comparing it with a standard undigested DNA sample. Serial dilutions were carried out to get desired quantity of DNA for polymerase chain reaction (PCR).

3.5.2.4 RAPD-PCR amplification

Reaction mixture was prepared in 0.2 ml thin walled PCR tubes containing the same components as mentioned for RAPD analysis of Fusarium oxysporum f. sp. lycopersici.

3.5.2.5 The thermoprofile for PCR

PCR amplification for RAPD analysis was performed according to Willams et al. (1990) with certain modifications. The optimum specifications followed for DNA amplification were as follows:

Step	Temperature (^oC)	Duration (min)	Number of cycles
Denaturation	94	4	1
Denaturation	94	1	
Annealing	37	1	
Extension	72	2	40
Final extension	72	7	1
Hold temperature	4	-	-

After completion of the PCR, products were stored at 4 ^oC until the gel electrophoresis was done.

Same solutions and primers as used for RAPD analysis of Fusarium oxysporum f. sp. lycopersici were employed.

The separation of amplified products by agarose gel electrophoresis and scoring of amplified fragments. Analyses of the profile of the amplified fragments were done same as mentioned above.

3.5.3 Detection of Meloidogyne incognita through amplification 18s rDNA gene

The DNA extraction was performed as described by Wood (1988).

3.5.3.1 Requirements

Stock solutions: Worm lysis buffer : (pH 8.5)

1% SDS

50 mM EDTA

100 mM NaCl

100 µg/ml proteinase K

1% 2-Mercaptoethonol

100 mM Tris-HCl

Phenol :Chloroform : Isoamyl alcohol-25:24:1

T_{10} E_1 : 10 mM Tris

1 mM EDTA

pH 8.0

3.5.3.2 Procedure

- Few nematodes were picked into microtube containing 500 µl worm lysis solution buffer
- Frozen at -80 ^oC followed by thawing and heating to 60 ^oC for 30 min.
- The supernatant were removed to a fresh tube, leaving behind nematode cuticular material.
- Equal volume of Phenol: Chloroform: IAA (25:24:1) was added in centrifugation tube, centrifuged at 10000 rpm for 10 min.

- Supernatant was taken in fresh centrifuge tube. To this, 1/10th volume of 3M sodium acetate and 0.54 volume of isoamyl alcohol was added. Mixed by gentle inversion and kept for 30 minutes at –20 °C.
- The DNA was recovered by centrifugation at 10000 rpm for 10 minutes at 4°C. The DNA pellet was washed with 70 per cent ethanol, air dried and re-suspended in 25-30 µ1 of $T_{10}E_{1.}$ This DNA obtained was further quantified by agarose gel electrophoresis.

3.5.3.3 PCR amplification

Requirements;

The total DNA isolated as described above was used as template DNA for reaction.

3.5.3.4 Primers

M. incognita 18s rDNA specific primers were designed by using Primer3 software. The designed primer pair is:

Primers	Sequence
Forward primer	5′-ATGTATAAGTTTAATCGTTTTAACGA -3′
Reverse primer	5′-GTATGTACCAACTATTTAGTAGGT- 3′

3.5.3.5 Standardization of PCR conditions

PCR conditions were standardized to amplify a single sharp amplicon by varying primer concentration, primer annealing temperature and the concentration of dNTPs. Different primer concentrations such as 2.5 pM, 5 pM and 10 pM were used for the purpose. PCR was performed to optimize reaction conditions. Based on this exercise, the following reaction mixture was employed for further studies.

3.5.3.6 Master mix for PCR

Reaction mixture was prepared in 0.2 ml thin walled PCR tubes containing the following components. The total volume of each reaction mixture was 20 µl. the following reaction mixture was found to be optimum for PCR amplification.

Taq buffer	:2.0 µl
dNTPs mix (2.5 mM each)	:2.0 µl
10x assay buffer with 15 mM $MgCl_2$	: 1.2 µl
Forword Primer (5 pM/ µl)	: 1.0 µl
Reverse primer (5 pm/ µl)	: 1.0 µl
Taq DNA polymerase (3.0U/ µl)	: 0.6 µl
Sterile distilled water	: 11.20 µl
Template DNA (25 ng/ µl)	: 1.0 µl
Total	: 20 µl

3.5.3.7 PCR programmes for amplification of 18s rDNA gene

Step	Temperature (°C)	Duration (min)	Number of cycles
Denaturation	94	7	1
Denaturation	94	1	
Annealing	66	1	
Extension	72	1.3	40
Final extension	72	10	1
Hold temperature	4	-	-

After completion, the samples were stored at 4^0C in a refrigerator for future use. Separation of amplified products by agarose gel electrophoresis same as described above reaction was done .using 1kb DNA ladder was used as reference.

3.6 SERODIAGNOSIS

3.6.1 Antiserum Production Against Fusarium oxysporum f. sp. lycopersici

3.6.1.1 Preparation of purified antigen from Fusarium oxysporum f. sp. lycopersici

The antigen was prepared from total protein extracted from mycelium of 7 days-old F. o. f. sp. lycopersici. The mycelial mat was filtered through a muslin cloth, washed twice with phosphate buffer saline (PBS; 0.8 % NaCl, 0.02% KCL, 0.115% Na_2 HPO_4, 0.025% KH_2PO_4, pH 7.2) and lyophilized. Lyophilized mycelium (1 g) was ground with liquid nitrogen and phosphate buffer saline (PBS; 10 ml) containing 0.1% cystein, 0.1% ascorbic acid, and 17% sucrose was added to the ground mycelium (El-Nashaar et al., 1986). The suspension was centrifuged at 10000 rpm for 30 min at 4 ^{o}C. The supernatant was concentrated over polyethylene glycol-6000 and then dialyzed against PBS (0.1 M, pH 7.2) for 24 h. Pellet was resuspended in 1 ml of PBS. The protein content was determined (Bradford, 1976) and adjusted to 1 mg/ ml in PSB and stored at -16 ^{o}C as antigen.

3.6.1.2 Preparation of antiserum:

Antiserum against F. o. f. sp. lycopersici was prepared using 3-month old female albino rabbit by giving four (weekly) intramuscular injections with purified preparation of the fungus suspended in PBS at concentration of 1mg/ml. For the first injection the purified fungus was emulsified with an equal volume of Freund´s complete adjuvant and for subsequent injections. Equal volumes of Freund´s incomplete adjuvant were used. The injections were administered at one-week interval. Two weeks after the last injection, rabbits were bled and the blood was collected from the marginal ear vein then left for two h at room temperature for clot formation. Collected blood was then kept in a refrigerator for overnight. The antiserum was clarified by centrifugation at 1500 rpm for 15 min at 4 ^{o}C and stored at -20 ^{o}C until further use (Srivastava and Arora, 1997).

3.6.2 Preparation of antigen and production antiserum against Ralstonia solanacearum

PAbs were developed in a three month-old female albino rabbit, using virulent fluidal bacterial whole cells in the presence of Freund's incomplete adjuvant (Robinson et al. 1995). For the preparation of the antigen, 48 h -old virulent colonies of R. solanacearum, irregularly round fluidal, pinkish-white colonies were isolated on TZC medium. R. solanacearum colonies were inoculated to 100 ml nutrient broth in 250 ml Erlenmeyer flasks and incubated at 32 °C. After 48 h, the bacterium was isolated from the suspension by centrifuging at 3000 rpm for 10 min. Bacterial cells (1 x10^4 cfu /ml) were injected at multiple sites subcutaneously. Eight such intermittent inoculations were given at an interval of four weeks. A final booster was given at 4^{th} week and rabbit was bled from the ear vein. Collected blood was allowed to clot at room temperature for 30 min followed by an incubation period of 2 to 3 h at 4 °C. The clot was separated by centrifugation at 1500 rpm for 15 min and antisera were collected and stored at -20 ^{o}C until further use.

3.6.3 Antiserum production against Meloidogyne incognita

3.6.3.1 Preparation of antigens

Vermiform preparasitic J_2 and eggs were collected and washed several times with sterile water and were suspended in 2.5 volumes of 0.05 M potassium phosphate buffer (pH 7.2) containing 0.14 N sodium chloride and 0.001 M magnesium chloride. These suspensions were ground in a mortar and pestle. The preparations were centrifuged at 20000 rpm for 30 min and the supernatants were stored at -20 ^{0}C and used as antigen.

3.6.3.2 Preparation of antisera

Antisera were produced in female albino rabbit. Four weekly intramuscular injections with antigen preparations (homogenates of J_2 and eggs,1mg/ml) were given: Antigen were emulsified with an equal volume of Freund´s complete adjuvant for subsequent injections the antigen was emulsified with an equal volume of Freund´s incomplete adjuvant. Two weeks after the last injection, rabbits were bled and the blood was collected from the marginal ear vein. Collected blood was left for two h at room temperature for clot formation, then the clot was separated sera were kept in a refrigerator for overnight. The antiserum was clarified by centrifugation at 1500 rpm for 15 min at 4 ^{o}C and stored at -20 ^{o}C until further use. (Charudattan and De vay,1972).

3.6.4 Tube precipitation test

With the view to determine the titre, tube precipitation test was performed. Antisera were diluted using two- fold dilution series in test tubes. To all the test tube, 0.5 ml aliquots of antigen were pipetted and the tubes were incubated at 37 ^{o}C in water both for four h. Phosphate buffer saline served as control in each series.

The amount of precipitation was evaluated according to the following scale, after examining under the microscope.

- : No precipitation
+ : slight precipitate
++ : moderate precipitate
+++ : heavy precipitate
++++ : very heavy precipitate

3.6.5 Purification of immonoglobulins (IgG)

The method described by Clark and Adams (1977) was employed for the purification of IgG from polyclonal antiserum.

Polyclonal antiserum of 1ml raised against F. o f. sp. lycopersici was added to 9 ml of distilled water and mixed thoroughly. To the diluted antiserum 10 ml of saturated ammonium sulphate was added and incubated for 30 min at room temperature. The mixture was centrifuged at 12000 rpm for 10 min at 4^{0}C. The pellet was dissolved in 2 ml of half the strength of phosphate buffer saline (PBS). The antibodies were dialysed for 24 h against half strength PBS, 500 ml per change and three such changes were given at an interval of 8 h. The immunoglobulins were further purified by passing through cellulose column.

3.6.5.1 Cellulose column

Approximately 10 ml of cellulose slurry prepared in the half strength phosphate buffer saline (PBS) was poured down from the sides of the column to avoid air bubbles and the cellulose was allowed to settle. The column (Plate 1) was calibrated by washing with half strength PBS (pH 7.4) till the pH of washing buffer matched with the pH of effluent. The liquid level was never allowed to drain at the top of the cellulose and the liquid was added carefully drop-wise to avoid disturbance to the column. The salt fractioned serum was then added drop wise to the precalibrated cellulose column. The stop cock valve was opened and the salt fractioned serum was allowed to run through the column. An additional 10 ml of half strength PBS was carefully added to the column. Effluent 1 ml aliquot was collected in pre-marked microfuge tubes until all 12 fractions were collected. The fractions were monitored separately, at 280 nm in a spectrophotometer using half strength PBS as a blank. The tubes with OD_{280} values above 1.4 were pooled and strength of IgG was adjusted to 1.4 OD using half strength PBS which resulted 1 mg/ml concentration.

3.6.6 ELISA ASSAY

3.6.6.1 DAC-ELISA

Direct antigen coating (DAC) ELISA was performed as per the procedure of Clark and Adams (1977).

- The test antigen was added to ELISA plate 200µl/well in 15 mM sodium bicarbonate buffer at pH 9.6 and plates were incubated at 4 ^{o}C over night.

Plate 1. Cellulose column used for purification of IgG

- The contents of the plates poured off and rinsed in PBS-Tween (PBS containing 0.5% Tween-20).This was followed by washing the plates in three changes of PBS-Tween buffer (200 µl/well) allowing 3 min. for each wash.
- Then the wells were blocked with 200 µl of ELISA blocking buffer (PBS containing 5% skimmed milk powder), the plates were incubated at 28 ^{o}C for 1 h and were washed in PBS-Tween as in step-2.
- Polyclonal antisera at 1:10000 in ELISA dilution buffer (PBS containg 0.1%BSA) was used at the rate of 100 µl/ well. Incubated 1 h at 37 ^{o}C.
- After washing, the secondary Ab-alkaline phosphatase labeled antirabbit was added at 1:2000 in ELISA dilution buffer and incubated for 1 h.
- Substrate buffer containing 0.5 mg/ml of p-nitro-phenyl phosphate (PNPP) was then dispenced to each well at the rate of 100 µl/well and incubated at room temperature for 30 min until sufficient colour developed.
- Results were assessed visually and quantified by reading the absorbance at 405 nm using a micro –titre plate reading. (Bioteck ELx 800 ELISA reader)

3.6.6.2 Double antibody sandwich enzyme linked immuno sorbent assay (DAS-ELISA)

The ELISA test for serodiagnosis requires purified immunoglobulins, (homologous antibodies) and enzyme conjugate. IgG was purified as described in the procedures 3.6.5 and 3.6.2.3 and conjugate was prepared by single step glutaraldehyde method.

3.6.2.3 Preparation of antibody enzyme conjugate

The one step glutaraldelyde method (Clark and Adams, 1977) was followed for preparation of alkaline phosphatase homologous conjugate (F. o. f. sp lycopersici -IgG^{*AP}).

Purified 1ml IgG (1 mg/ml) was mixed with 50 µl of enzyme alkaline phosphatase and made 0.05 per cent final concentration with fresh EM grade glutaraldehyde. The mixture was incubated at room temperature for four h till a very faint brown colour developed. The mixture was dialysed against three changes of 500 ml half strength PBS at 8 h interval. After dialysis, Bovine serum albumin was added to obtain a concentration of 5 mg per ml and stored at 4^0C.

3.6.2.4 Double –antibody sandwich (DAS) ELISA procedure

- 200 µl aliquots of purified IgG diluted in coating buffer dispended into each well of microtitre plate and incubated at 37 °C for 3h.
- The contents of the plates were poured off and rinsed in PBS-Tween. This was followed by washing the plates in three changes of PBS-Tween buffer allowing 3 min. for each wash.
- The purified antigen in phosphate buffer was dispensed into each well of microtitre plate at the rate of 200 µl per well and the plates were incubated at 37 °C for 3 h. And phosphate buffer saline served as negative control. After incubation period, content were poured off and plate was washed as described in step-2
- To each well 200 µl of F. o f. sp lycopersici -IgG^{*AP} (homologous conjugate) was added and the plates were incubated at 4 °C overnight and plates were washed with PBS-Tween buffer as mentioned earlier.
- Substrate buffer containing 0.5 mg/ml of p-nitro phenyl phosphate (PNPP) was then dispensed to each well at the rate of 200 µl/well and incubated at room temperature for 10-30 min. Light orange to yellow color, development indicated a weak to strong positive reaction and were quantitatively recorded in Biotek EL_X 800 ELISA reader at 405 nm.

Purification of immonoglobulins (IgG), Preparation of antibody anzyme conjugate and DAS- ELISA procedure for Ralstonia solanacearum were followed as mentioned for Fusarium oxysporum f. sp. lycopersici..

3.6.3 Sensitivity of the Direct- ELISA technique for the detection pathogens affecting tomato from plant and soil samples

Soil and plant samples used in this study were collected directly from the naturally infested field. 100g infested tomato rhizosphere soil sample was taken in a 250 ml flask containing 100ml of 0.1 M PBS pH 7.2, ten ml of this mixture were taken and centrifuged at 1000 rpm for 10 min. Supernatants were collected and used as antigen. Soil collected from healthy tomato rhizophere soil was used as a control. This was a combination of technique used by Janse (1988) and Seal et al. (1992).

Plant samples were prepared from wilt infected tomato plant (vascular region) were gound for 5 min using a blender containing 100 ml 0.1 M PBS buffer. Ten ml of this plant exctract was taken and centrifuged at 1000 rpm for 10 minutes. The supernatant was resuspended in 0.1 M PBS (pH 7.2) and used as source of antigen. Healthy tomato plant used as control.

4. EXPERIMENTAL RESULTS

The present investigation on molecular and serological diagnosis of vascular pathogens (Fusarium oxysporum. f. sp. lycopersici, Ralstonia solanacearum and Meloidogyne sp.) affecting tomato, was conducted in Department of Plant Pathology and Institute of Agri-Biotechnology (IABT), College of Agriculture, University of Agricultural Sciences, Dharwad during 2007 to 2010. The results of the experiments conducted are presented hereunder.

4.1 Collection of diseased samples

Vascular pathogens affecting tomato (F. o. f. sp. lycopersici and R. solanacearum) samples were collected during the year 2008-2009 from different tomato growing regions of Karnataka, viz. Dharwad (UAS Dharwad, Saidapur, Garag and Saptapur), Bangalore (Nelamangala, Doddaballapur and UAS Bangalore), Tumkur (Gubbi and Hosalli), Gadag (Bannikoppa and Lakkundi), Haveri, (Hangal, Chalageri and Ranebennur), Belgaum (Arabhavi, Khanapur and Gokak), Mysore (Hunsur), Chikkaballapur (Chikkaballpur and Chintamani), Shimoga (Shimoga and Sagar) Ramanagar, Kolar, Chikkamanglur and Davanagere. The wilted plants were collected and the pathogens were isolated from each location was designated as isolates viz. Fol-1 to Fol-23 of F. o. f. sp. lycopersici (Plate 2). And Rs-1 to Rs-24 of R. solanacearum (Plate 3).

The wilt disease is further aggravated in presence of nematode M. incognita, the latter alone causes root knot disease. Such root knot samples were collected from Dharwad (UAS Dharwad, Saidapur), Belgaum (Arabhavi), Bangalore (UAS Bangalore). Four cultures were used for further investigations.

4.2 Symptomatology

4.2.1 Fusarium wilt

Infected plants are stunted, the older leaves droop and curve downward and the plant frequently wilt and die. Symptoms on older plants generally become apparent during the interval from blossoming to fruit maturation. Earliest symptoms observed were yellowing of older and lower leaves. The yellowing process gradually increases and accompanied by wilting (Plate 4a) of the plant during the hottest part of day. The wilt becomes more extensive from day to day until the plant collapses The vascular tissue of a diseased plant turn dark brown (Plate 4b). The browning of the vascular tissue is characteristic of the disease and can be used for its tentative identification.

4.2.2 Bacterial wilt

Bacterial wilt caused by R. solanacearum is a serious soil borne disease of tomato. The initial symptom in mature plants under natural condition is wilting of upper leaves during the hottest part of the day followed by recovery during the evening and early hours of morning. Under favorable condition complete wilt occurs (Plate 5a).

The vascular tissues in the lower stem of wilted plants show a dark brown discoloration. These symptoms are similar to those of some fungal diseases. A cross section of stem of the plant with bacterial wilt produces a white, milky strand of bacterial cells in clear water (Plate 5b). This ooze distinguishes the wilt caused by the bacterium from that caused by fungal pathogen.

4.2.3 Root knot

Infected tomato plants were stunted in their growth with yellow coloured leaves (Plate 6a). When infected plants were uprooted, deformed roots with prominent galls of varying size were noticed (Plate 6b). Microscopic examination of the galls revealed the presence of egg, juveniles and females of M. incognita in the vascular region of roots. The presence of egg masses outside the gall was a common phenomenon noticed in tomato plant.

Plate 2. Isolates of Fusarium oxysporum f. sp. lycopersici in major tomato growing areas of Karnataka

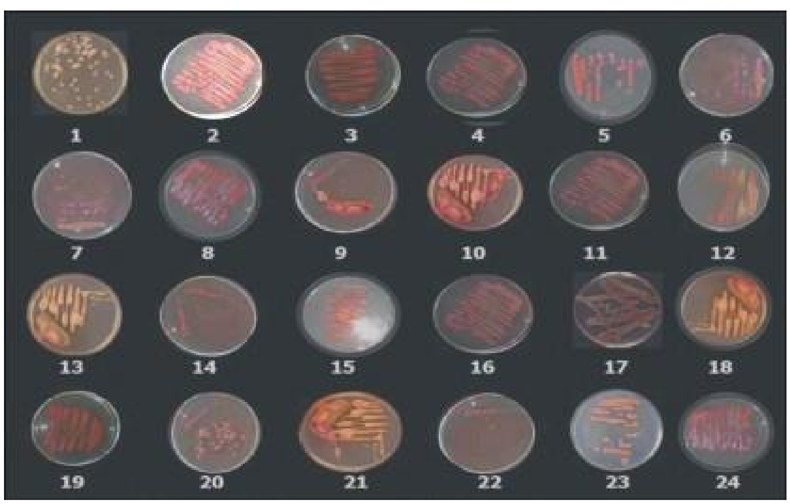


Plate 3. Isolates Ralstonia solanacearum in major tomato growing areas of Karnataka

a. Yellowing and wilting b. Vascular discolouration

Plate 4. Symptoms of Fusarium wilt of tomato

a. Green wilt symptom b.Ooze test for confirmation of bacterial wilt

Plate 5. Symptoms of bacterial wilt of tomato

a.Yellowing b.Root galls

Plate 6. Symptoms of Meloidogyne incognita infected tomato plant

4.3 Isolation and identification of Fusarium oxysporum. f. sp. lycopersici and study of morphological, cultural and pathogenic characters

4.3.1 Isolation

Isolation from the root and collar region of wilted tomato plant samples collected from different locations yielded 23 isolates of F. o. f. sp. lycopersici .These isolates were named after the place from which the plant samples were collected. Individual single spore cultures established from all the isolates were maintained on PDA by periodic transfer to fresh medium.

4.3.2 Identification of Fusarium oxysporum. f. sp. lycopersici

The morphological characters of F. o. f. sp lycopersici observed were all most similar to those described by Gerlach and Nirenberg (1982). The mycelia of pathogen were white cottony to pink. Often with purple tinge or reddish coloration on PDA medium. Microconidia were abundant, hyaline, oval-ellipsoid, straight to curved, where as macroconidia were hyaline, sparse to abundant, three to five septate, fusoid-subulate and pointed at both ends, have pedicellate base. However three septate spores were predominant. Chlamydospores, both smooth and rough walled which were abundant and form terminally or on an intercalary basis.

4.3.3 Grouping of isolates of Fusarium oxysporum. f. sp. lycopersici based on morphological, cultural characters and pathogenicity

Studies on morphological and cultural characters of twenty three isolates were carried out on potato dextrose agar as described in “Material and Methods”. The cultural characters were taken into account for assessing the existence of variation in isolates. The colony diameter, colony characters, sporulation, septation and spore size of fungus was recorded when maximum growth was attained on any one of the isolates (Table 3).

The 23 isolates of F. o. f. sp lycopersici were classified into three groups (Table 4). The first group isolates showed abundant aerial mycelium, initially white, cottony, fluffy, turned to pale pink to purple, chlamydospores round to slightly elliptical, single or two celled, with abundant sporualation and maximum colony diameter ranging between 75.0 to 90.0mm.Group second isolates showed, moderate to abandant aerial mycelium, cottony, fluffy, white to pale pink with good sporulation, chlamydospores terminal or intercalary, one or two celled circular to oval, colony diameter varies from 60.0 to 90.0mm. Third group isolates exhibited cottony aerial mycelium, fluffy, white to pale pink, sporulation moderate to poor, one or two celled, terminal or intercalary chlamydospores, colony diameter ranged between 55.0 to 90.0mm.

The pathogencity test of all isolates was confirmed on tomato cv. Pusa ruby. Plants inoculated with spore suspension of fungus started expression of wilting symptoms within two weeks after inoculation (Plate 7). Eight isolates (Fol-1, Fol-4, Fol-6 Fol-9, Fol-11, F ol-13, Fol-15 and Fol-21) caused typical symptoms of Fusarium wilt and aggressiveness of the isolates was variable but generally high. They showed strong virulence with 75 per cent severity and wilting symptoms were noticed 14 days after inoculation. Group second isolates viz., Fol-2, Fol-3, Fol-5,Fol-7,Fol-19,Fol-22 were the next best virulent by producing wilt symptoms in 17 to 21 days after inoculation, showed 53-75 per cent severity. And third group comprising Fol-8, Fol-10, Fol-12, Fol-14, Fol-16, Fol-17, and Fol-18, Fol-20 and Fol- 23 isolates were less aggressive and took more time to cause symptoms (24-30 days) and severity varied from 26-50 per cent. Reisolations from diseased plant consistently yielded the respective isolates of F. o. f. sp. lycopersici.

Table 3. Morphological, cultural and pathogenic variability in isolates of Fusarium oxysporum f. sp. lycopersici on potato dextrose agar

Isolates	Growth characters	Colony diameter (mm)	Sporulation	Micro conidia	Macro conidia	Disease severity index (%)	Location
Fol-1	Abundant aerial mycelium, initially white, cottony, fluffy, turned to pale pink	90.00	++++	Fusiform with 1 septa (11.1-13.4x 4.4 -5.6 µm)	Fusiform with 5 septa (27.8-33.5 x 3.3-5.0 µm)	75.0	Saidapur
Fol-2	Modarate aerial mycelium, cottony, fluffy, white to pale pink	65.00	+++	Fusiform with 1 septa (10.0-12.5x 4.1 -5.3 µm)	Fusiform with 3 septa (26.5-33.5 x 3.4-4.8 µm)	53.0	Garag
Fol-3	Modarate aerial mycelium, cottony, fluffy, white to pale pink	88.00	+++	Fusiform with 1septa (12.2-13.6 x 3.1-4.0 µm)	Fusiform with 5 septa (35.2-37.4 x 3.3-5.0 µm)	65.0	Nelamangala
Fol-4	Abundant aerial mycelium, initially white, cottony, fluffy, turned to pale pink	88.00	++++	Fusiform with 1septa (12.0-13.8 x 3.4-4.0 µm)	Fusiform with 5 septa (39.2-42.0 x 4.3-4.9 µm)	75.0	Doddaballapur
Fol-5	Modarate aerial mycelium, cottony, fluffy, white to pale pink	75.00	+++	Oval round (6.5-7.3x3.5-3.8 µm)	Fusiform with 3 septa (28.5-35.4 x 3.4-5.3 µm)	75.0	Gubbi
Fol-6	Abundant aerial mycelium, initially white, cottony, fluffy, turned purple	85.00	++++	Oval round (5.3-5.4 x 2.3 -3.5 µm)	Fusiform with 5 septa (32.0-35.2 x 3.9-5.0 µm)	75.0	Hosalli
Fol-7	Abundant aerial mycelium, cottony, fluffy, white to pale pink	60.00	+++	Oval round (5.5-6.3x3.2-3.5 µm)	Fusiform with 5 septa (39.0-41.0 x 3.4-5.0 µm)	26.0	Bannikoppa
Fol-8	Abundant aerial mycelium, cottony, fluffy, white to pale pink	88.00	++	Fusiform without septa (11.0-13.5x 4.3 -5.5 µm)	Fusiform with 3 septa (28.2-31.0 x 3.5- 3.8 µm)	30.0	Lakkundi
Fol-9	Abundant aerial mycelium, initially white, cottony, fluffy, turned to pale pink	90.00	++++	Oval round (6.5-7.4 x 3.0-3.5 µm)	Fusiform with 5 septa (38.0-40.0 x 3.5-4.2 µm)	75.0	Hangal
Fol-10	Abundant aerial mycelium, cottony, fluffy, white to pale pink	58.00	++	Fusiform with 1septa (12.4-13.8x3.5-4.0 µm)	Fusiform with 5 septa (40.0-42.3 x 3.8-4.9 µm)	75.0	Ranebennur
Fol-11	Abundant aerial mycelium, initially white, cottony, fluffy, turned to pale pink	75.00	++++	Fusiform with 1septa (11.5-14.8 x 3.5-4.0 µm)	Fusiform with 3 septa (25.3-26.6 x 3.3-5.4 µm)	45.0	Arabhavi
Fol-12	Modarate aerial mycelium,cottony, fluffy, white to pale pink	65.00	++	Oval round 5.6-6.0 x 3.3 -3.7 µm.	Fusiform with 3 septa (26.2-28.3 x 3.9-4.8 µm)	45.0	Khanapur
Fol-13	Abundant aerial mycelium, initially white, cottony, fluffy, turned to pale pink.	87.00	++++	Fusiform with 1septa (12.3-14.2 x 3.6-4.6 µm)	Fusiform with 5 septa (34.0-35.6 x 3.6-4.8 µm)	75.0	Gokak
Fol-14	Modarate aerial mycelium, cottony, fluffy, white to pale pink	85.00	+	Oval round (5.4-5.7 x 3.4 -3.8 µm)	Fusiform with 3 septa (25.2-27.3 x 3.4-5.0 µm)	35.0	Mysore
Fol-15	Abundant aerial mycelium, initially white, cottony, fluffy, turned to pale pink to purple	88.00	++++	Fusiform with 1septa (10.8-13.5x 3.7-4.0 µm)	Fusiform with 3 septa (26.2-27.3 x 3.3-4.5 µm)	75.0	Hunsur
Fol-16	Modarate aerial mycelium, cottony, fluffy, white to pale pink	85.00	+	Fusiform without septa (10.5-12.2 x 3.4 -4.8 µm)	Fusiform with 5 septa (27.2-28.3 x 3.4-4.5 µm)	45.0	Chikkballapur

Contd…

Isolates	Growth characters	Colony diameter (mm)	Sporulation	Micro conidia	Macro conidia	Disease severity index (%)	Location
Fol-17	Modarate aerial mycelium, cottony, fluffy, white to pale pink	65.00	++	Fusiform with 1 septa (12.1-14.4 x 3.5-4.0 µm)	Fusiform with 3 septa (28.3-32.4 x 3.3-5.0 µm)	50.0	Chintamani
Fol-18	Modarate aerial mycelium, cottony, fluffy, white to pale pink	80.00	++	Oval round (5.5-5.7 x 3.3 -3.7 µm)	Fusiform with 3 septa (24.5-25.5 x 3.8-4.6 µm)	35.0	Shimoga
Fol-19	Abundant aerial mycelium, cottony, fluffy, white to pale pink	85.00	+++	Oval round (5.3-6.1x3.0-3.3 µm)	Fusiform with 3 septa (26.7-28.4 x 3.8-5.0 µm)	69.0	Sagar
Fol-20	Modarate aerial mycelium, cottony, fluffy, white to pale pink,	86.00	++	Fusiform with 1septa (12.4-13.7 x 3.7-5.0 µm)	Fusiform with 5 septa (42.0-43.5 x 3.8-5.0 µm)	30.0	Ramanagar
Fol-21	Abundant aerial mycelium, initially white, cottony, fluffy, turned to pale pink to purple	90.00	++++	Fusiform without septa (9.2-16.4 x 2.9-4.0 µm)	Fusiform with 3 septa (27.6-31.5 x 3.4-5.0 µm)	75.0	Kolar
Fol-22	Abundant aerial mycelium, cottony, fluffy, white to pale pink	76.00	+++	Fusiform with 1septa (12.5-13.5x 4.4 -5.0 µm)	Fusiform with 5 septa (37.3-40.1 x 3.8-5.0 µm)	73.0	Chikkmangalu r
Fol-23	Modarate aerial mycelium, cottony, fluffy, white to pale pink	85.00	+	Fusiform with 1septa (12.1-13.4x 4.4 -5.0 µm)	Fusiform with 5 septa (35.0-38.2 x 4.7-5.0 µm)	50.0	Davanagere

++++ = Abundant sporulation = > 80 number of conidia/microscopic field
+++ = Good sporulation = 50 - 80 number of conidia/microscopic field
++ = Modarate sporulation = 25-50 number of conidia/microscopic field
+ = Spare sporulation = <25 number of conidia/microscopic field

0 = No symptoms
1 = <25% leaves with symptoms
2 = 26-50% leaves with symptoms
3 = 51-75% leaves with symptoms
4 = 76-100 leaves with symptoms

Table 4. Grouping of Fusarium oxysporum f. sp. lycopersici isolates on the basis of morphological, cultural and pathogenicity

Group	Isolates	Morphological and cultural characters on PDA	Microconidia	Macroconidia	Disease severity index (%)	Location
Group I	Fol-1 Fol-4 Fol-6 Fol-9 Fol-11 Fol-13 Fol-15 Fol-21	Abundant aerial mycelium, initially white, cottony, fluffy, turned to pale pink to purple, chlamydospores round to elliptical : single or two celled, sporualation abundant, colony diameter 75-90mm.	Oval round to fusiform, 0-1septate. (5.3-5.4 x 2.3 -3.5 to 12.4-13.8x3.5-4.0 µm)	Fusiform with 1-5 septa,mostly 3 septa, (25.3-26.6 x 3.3-5.4 to38.0-40.0 x 3.5-4.2 µm)	75.0	Saidapur Doddaballapur Hosalli Hangal Arabhavi Gokak Hunasur Kolar
Group II	Fol-2 Fol-3 Fol-5 Fol-7 Fol-19 Fol-22	Modarate to abandant aerial mycelium, cottony, fluffy, white to pale pink, good sporulation, chlamydospores terminal or intercalary, one or two celled circular to oval, colony diameter 60-90mm.	Oval round to fusiform, 0-1septa (5.3-6.1x3.0-3.3 µm to12.5-13.5x 4.4 -5.0 µm)	Fusiform with 1-5 septa, mostly 3 septa (26.5-33.5 x 3.4-4.8 to37.3-40.1 x 3.8-5.0 µm)	53-75	Garag Nelamangala Gubbi Bannikoppa Sagar Kolar Chikkmanglur
GroupIII	Fol-8 Fol-10 Fol-12 Fol-14 Fol-16 Fol-17 Fol-18 Fol-20 Fol-23	Modarate to abandant aerial mycelium aerial mycelium, cottony, fluffy,white to pale pink,sporulation moderate to poor, chlamydospores terminal or intercalary,one or two celled, colony diameter 58-90mm	Oval round to fusiform, 0-1septa(5.5-5.7 x3.3 -3.7 to 12.4-13.7 x 3.7-5.0 µm)	Fusiform with 1-5 septa, mostly 3 septa,(24.5-25.5 x 3.8-4.6 to42.0-43.5 x 3.8-5.0 µm)	26-50	Lakkundi Ranebennur Khanapur Chikkballapur Chintamani Shimoga Ramanagar Davanagere

Abundant sporulation = > 80 conidia/microscopic field — 1 = <25% leaves with symptoms
Good sporulation = 50 - 80 conidia/microscopic field — 2 = 26-50% leaves with symptoms
Modarate sporulation = 25-50 conidia/microscopic field — 3 = 51-75% leaves with symptoms
Spare sporulation = <25 conidia/microscopic field — 4 = 76-100 leaves with symptoms

4.4 Isolation and identification of Ralstonia solanacearum from wilted tomato plants

Isolation of the bacterium was made from the tomato plants showing typical symptoms of wilts, from major tomato growing areas belonging to different agro-climatic zones of Karnataka by following dilution plate technique on TZC medium.

The virulent colonies from each location were picked up and purified on TZC agar. The purified colonies were stored in sterile distilled water contained in small polyprolene culture tubes at -21^{0}C.

The isolated bacterium was subjected to various molecular and serological tests. All the isolates produced fluidal, slimy, irregular to round, and dull white with pink to reddish center (Table 5) on TZC medium after 48 h incubation at 32^{o}C.

Isolates were similar in their appearance under microscope and were found to be small rods occurring singly, rarely in pairs. In gram staining study reveled that all the isolates were gram negative.

4.4.1 Pathogenicity Test

The pathogenicity of all isolates was determined by inoculating a bacterial suspension (1X10^{-8}cfu/ ml) into susceptible tomato (Pusa ruby) plants at preferably the third true leaf stage.

Artificially inoculated plants started expressing wilt symptoms within 15 days as similar symptom was observed as described in symptomatology, re-isolation of the causal organism was made from the artificially inoculated wilted plants and were similar to the original culture with respect to their morphology and cultural characters (Plate 8).

Out of twenty four, twelve isolates (Rs-1, Rs-4, Rs-7, Rs-8, Rs-9, Rs-12, Rs-16, Rs-19, Rs-21, Rs-22 Rs-23 and Rs-24) were highly virulent collected from, UAS Dharwad, UAS Bangalore, Hosalli, Bannikoppa, Lakkundi, Arabhavi, Hunsur, Shimoga, Ramanagar, Kolar, Chikkmangalur and Davanagere. Where as isolates (Rs-2, Rs-5, Rs-6, Rs-10, Rs-11 and Rs-20) collected from Garag, Doddaballapur, Gubbi, Ranebennur, Chalageri and Sagar showed medium virulence. Isolates (Rs-3, Rs-13, Rs-14, Rs-15, Rs-17 and Rs-18) collected from Sapthapur, Gokak, Khanapur, Mysore, Chikkballapur and Chintamani showed low virulence as compaired to above two group.

4.5 Collection, identification and pathogencity of root knot nematode

The collection, maintaince and identification of root knot was carried out as explained in ‘Material and Methods’

Pathogencity of all collected isolates were proved by inoculating nematode suspension. The artificially inoculated plant showed stunted growth with yellow coloured leaves. When infected plants were uprooted, deformed roots with prominent galls of varying size were noticed (Plate 9).

4.6 Molecular diagnosis

4.6.1 RAPD analysis of Fusarium oxysporum. f. sp. lycopersici

It is difficult to distinguish these isolates using traditional morphological differences. Random amplified polymorphic DNA (RAPD) was used to detect the variation among the isolates of F. o. f. sp. lycopersici. OPA, OPB and OPF series primers were used to determine genetic distance between isolates and to construct a dendrogram. Banding profile of different primers for twenty three isolates of F. o. f. sp. lycopersici is given in Table 6 and Plate 10.

Of the 30 primers used for amplification, OPA5, OPA8, OPA 10, OPB5, OPB7, OPB8, OPF2 and OPF9 showed 100 per cent polymorphism. A total of 331 amplicon levels resulted from 30 primers were available for analysis. A total of 75.09 per cent polymorphism was found among the isolates indicating there is a high molecular variability among the isolates

Table 5: Colony characteristics and relative pathogencity of isolates of Ralstonia solanacearum obtained from different agroclimatic zones of Karnataka

Isolates	Cultural characteristics on TZC medium	Pathogenic reaction of isolates	
		Relative pathogencity	Score
Rs-1	Round, dull white with slight red center	High	4.8
Rs-2	Fluidal, irregular to round, dull white, with light pink center	Medium	3.6
Rs-3	Irregular, dull white, with light pink center	Low	2.2
Rs-4	Fluidal, irregular to round , dull white with slight pink center	High	4.7
Rs-5	Irregular to round, dull white with slight pink center	Medium	3.2
Rs-6	Round, dull white with slight red centers	Medium	3.4
Rs-7	Irregular to round, dull white with slight red center	High	4.6
Rs-8	Irregular to round, dull white with slight pink center	High	4.4
Rs-9	Fluidal, irregular, dull white with slight pink center	High	4.8
Rs-10	Fluidal, irregular, dull white with slight pink center	Medium	3.5
Rs-11	Fluidal, irregular, dull white with slight pink center	Medium	3.3
Rs-12	Irregular dull white with slight pink center	High	4.8
Rs-13	Irregular dull white with slight pink center	Low	2.3
Rs-14	Fluidal, irregular to round, dull white, with light pink center	Low	2.0
Rs-15	Round, dull white with slight red center	Low	2.1
Rs-16	Irregular dull white with slight pink center	High	4.6
Rs-17	Irregular dull white with slight red center	Low	2.3
Rs-18	Fluidal, irregular, dull white with slight pink center	Low	2.4
Rs-19	Irregular to round, dull white with slight red center	High	4.5
Rs-20	Fluidal, irregular, dull white with slight pink center	Medium	3.4
Rs-21	Round, dull white with slight red center	High	4.7
Rs-22	Fluidal, irregular, dull white with slight pink center	High	4.7
Rs-23	Irregular to round, dull white with slight red center	High	4.5
Rs-24	Irregular dull white with slight pink center	High	4.7

1= no visible symptoms
2=1-25 % of the plant is wilting
3=26-50% wilt
4=51-75% wilt
5= more than 75% wilt

Inoculated Uninoculated

Plate 7. Proving pathogenicity of *Fusarium oxysporum* f. sp. *lycopersici*

Inoculated Uninoculated

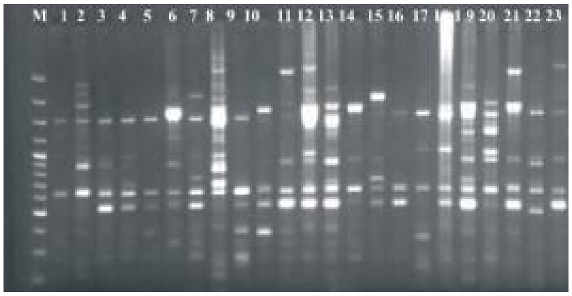
Plate 8. Proving pathogenicity of *Ralstonia solanacearum*

Inoculated Root galls Uninoculated

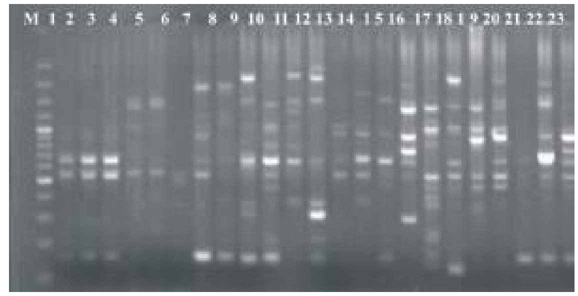
Plate 9. Proving pathogenicity of root knot nematode

Table 6. Banding profile of different primers for different isolates of Fusarium oxysporum f. sp. lycopersici

Primers	Total bands	Polymorphic bands	% Polymorphism
OPA 1	11	7	63.63
OPA2	12	9	75.00
OPA3	11	7	63.63
OPA4	11	8	72.72
OPA5	16	16	100.00
OPA6	12	9	75.00
OPA7	9	8	88.88
OPA8	16	16	100.00
OPA9	13	10	76.92
OPA10	10	10	100.00
OPB1	12	8	66.66
OPB2	15	14	93.33
OPB3	13	6	46.15
OPB4	15	5	33.33
OPB5	8	8	100.00
OPB6	10	9	90.00
OPB7	9	9	100.00
OPB8	12	12	100.00
OPB9	13	12	92.30
OPB10	11	6	54.54
OPF1	8	7	87.50
OPF2	6	6	100.00
OPF3	16	10	62.50
OPF4	12	6	50.00
OPF5	15	6	40.00
OPF 6	14	5	38.46
OPF7	9	4	44.44
OPF8	5	3	60.00
OPF9	7	7	100.00
OPF10	9	7	77.77
Total	331	250	75.09



OPA 3



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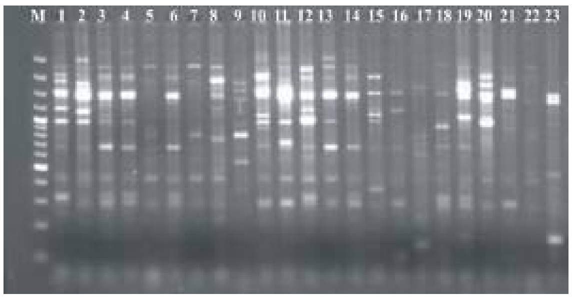

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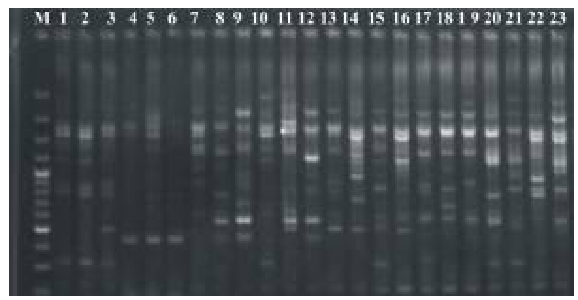

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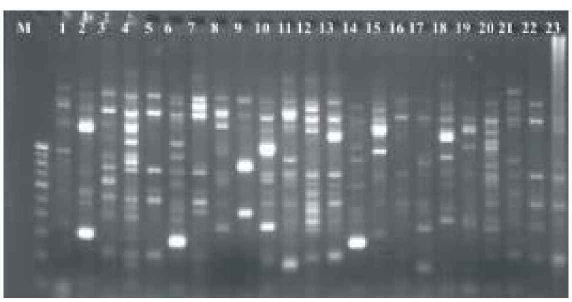
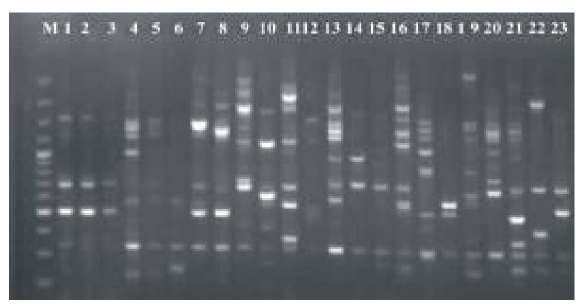


OPA 4



OPA 6

Plate 10. RAPD banding pattern in Fusarium oxysporum f. sp. lycopersici isolates

Table 7. Similarity coefficient of twenty three isolates of Fusarium oxysporum f. sp. lycopersici

Isolates	Fol-1	Fol-2	Fol-3	Fol--4	Fol-5	Fol-6	Fol-7	Fol-8	Fol-9	Fol-10	Fol-11	Fol-12	Fol-13	Fol-14	Fol-15	Fol-16	Fol-17	Fol-18	Fol-19	Fol-20	Fol-21	Fol-22	Fol-23
Fol-1	1																						
Fol-2	0.64	1																					
Fol-3	0.60	0.67	1																				
Fol-4	0.51	0.52	0.63	1																			
Fol-5	0.47	0.51	0.58	0.73	1																		
Fol-6	0.49	0.56	0.67	0.59	0.59	1																	
Fol-7	0.45	0.48	0.56	0.59	0.57	0.43	1																
Fol-8	0.47	0.50	0.51	0.54	0.54	0.52	0.62	1															
Fol-9	0.47	0.47	0.48	0.48	0.56	0.39	0.68	0.56	1														
Fol-10	0.51	0.54	0.57	0.55	0.51	0.54	0.45	0.50	0.44	1													
Fol-11	0.48	0.55	0.52	0.48	0.45	0.55	0.48	0.52	0.52	0.59	1												
Fol-12	0.51	0.54	0.54	0.52	0.48	0.53	0.50	0.61	0.52	0.59	0.64	1											
Fol-13	0.53	0.55	0.59	0.53	0.52	0.58	0.47	0.54	0.44	0.56	0.60	0.65	1										
Fol-14	0.47	0.48	0.44	0.45	0.47	0.53	0.44	0.45	0.40	0.53	0.55	0.61	0.55	1									
Fol-15	0.52	0.52	0.49	0.47	0.48	0.49	0.45	0.49	0.43	0.52	0.51	0.55	0.50	0.57	1								
Fol-16	0.37	0.42	0.42	0.44	0.42	0.44	0.41	0.48	0.49	0.46	0.50	0.54	0.47	0.55	0.48	1							
Fol-17	0.39	0.35	0.38	0.43	0.44	0.41	0.42	0.44	0.50	0.42	0.39	0.38	0.44	0.43	0.44	0.51	1						
Fol-18	0.39	0.43	0.43	0.43	0.44	0.43	0.45	0.50	0.45	0.45	0.49	0.54	0.47	0.55	0.49	0.50	0.55	1					
Fol-19	0.37	0.40	0.44	0.45	0.47	0.48	0.39	0.47	0.40	0.48	0.52	0.54	0.56	0.52	0.53	0.51	0.52	0.57	1				
Fol-20	0.42	0.43	0.42	0.45	0.45	0.47	0.39	0.53	0.37	0.44	0.50	0.54	0.53	0.55	0.55	0.49	0.45	0.50	0.65	1			
Fol-21	0.36	0.40	0.43	0.43	0.43	0.45	0.44	0.52	0.43	0.48	0.54	0.59	0.57	0.55	0.53	0.58	0.42	0.52	0.62	0.59	1		
Fol-22	0.36	0.43	0.42	0.37	0.39	0.45	0.37	0.50	0.43	0.43	0.44	0.48	0.45	0.49	0.46	0.59	0.46	0.45	0.50	0.56	0.57	1	
Fol-23	0.41	0.44	0.53	0.43	0.45	0.49	0.42	0.47	0.43	0.47	0.52	0.53	0.50	0.59	0.56	0.53	0.45	0.53	0.61	0.52	0.59	0.58	1

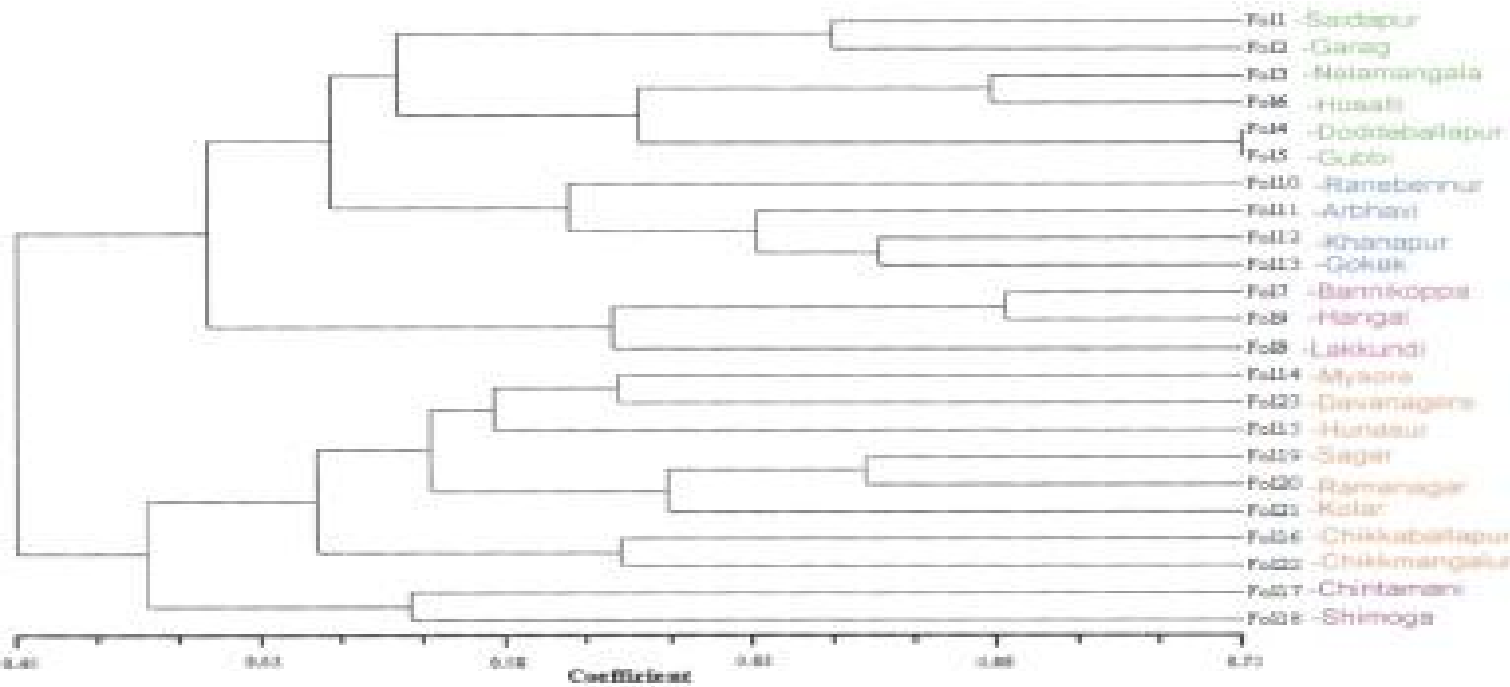


Fig. 1. Dendrogram for twenty three isolates of *Fusarium oxysporum* f. sp. *lycopersici* based on RAPD analysis

Fig1. Dendrogram for twenty three isolates of Fusarium oxysporum f. sp. lycopersici based on RAPD analysis

Based on simple matching coefficient, a genetic similarity matrix was constructed to assess the genetic relatedness among the isolates of F. o. f. sp. lycopersici. Genetic similarity coefficient of twenty three isolates of F. o. f. sp. lycopersici is given in the Table 7. Similarity coefficient ranged from 0.35 to 0.73. The maximum genetic similarity of 73 per cent was recorded between Gubbi (Fol-5) and Doddaballapur (Fol-4) isolates. Whereas, least genetic similarity was observed between Chintamani (Fol-17) and Garag (Fol-2) isolates followed by Lakkundi (Fol-9) and Hosalli (Fol-7) with 68 per cent similarity.

. Information on the banding pattern for all the primers was used to determine genetic distance between the isolates and to construct a dendrogram by using unweighted pair group arithmetic mean method (UPGMA)

The dendrogram for pooled data (of primers) showed five major clusters (Fig 1). The isolates Fol-1, Fol-2, Fol-3, Fol-6, Fol-4 and Fol-5 collected from Saidapur, Garag, Nelamangala, Hosalli, Doddaballapur and Gubbi locations were found in one cluster, isolates Fol-10, Fol-11, Fol-12, and Fol-13 from Ranebennur, Arabhavi, Khanapur and Gokak formed a separate cluster, Fol-7, Fol-9 and Fol-8, (Bannikoppa, Hangal, and lakkundi). Isolates were found in third cluster, Fol-14, Fol-23, Fol-15, Fol-19, Fol-20, Fol-21, Fol-16 and isolates Fol-22 (Mysore, Davanageri, Hunasur, Sagar, Ramanagar, Kolar and Chikkballapur) in fourth cluster and Fol-17 (Chintamani) and Fol-18 (Shimoga) isolates were found in fifth cluster.

4.6.2 RAPD analysis of Ralstonia solanacearum

RAPD was used to detect the variation among the 24 isolates of R. solanacearum collected from different district of Karnataka. OPA, OPB and OPF series of primers were used to determine genetic diversity between the isolates to construct a dendrogram.

The profile of amplicons of different primers for twenty four isolates of R. solanacearum is given in Table 8 and Plate 11. A total of 241 DNA bands were detected using 30 primers, total of the 139 bands were polymorphic. Out of 30 primers OPA 10, OPB3, OPF7 and OPF 8 showed 100 per cent polymorphism. The banding profile per primer also varied from minimum of 4 bands (OPA3) to maximum of 16 bands (OPF3). From the RAPD analysis, the results revealed that a total of 73.93 per cent polymorphism was found between the isolates, indicating there was a high molecular variability among the isolates.

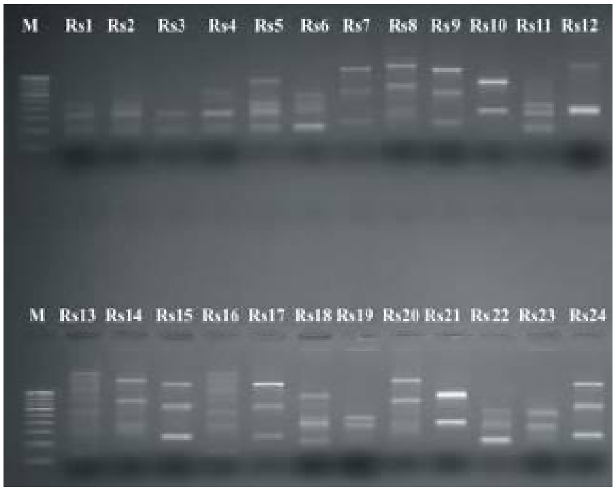
Based on the simple matching coefficient a genetic similarity matrix was constructed to access the genetic relatedness among the isolates (Table 9). The genetic similarity co-efficient of twenty four isolates based on RAPD analysis is given in Table, 8. The similarity co-efficient ranged from 0.19 to 0.61. The maximum genetic diversity of 0.61 percent was observed between Hosalli (Rs-7) and Doddaballapur (Rs-9) isolates, where as least similarity (0.19 per cent) was observed between Kolar (Rs-22) and Garag (Rs-2) isolates.

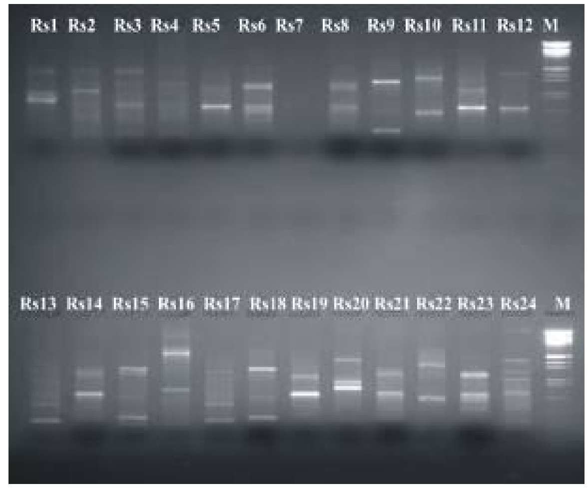
Information on the banding pattern for all the primers was used to determine genetic distance between the isolates and to construct a dendrogram by using unweighted pair group arithmetic mean method (UPGMA).

Further, the dendrogram constructed by UPGMA from the pooled data clearly showed two major clusters viz., A and B at similarity co-efficient of 0.29. The dendrogram for pooled data showed seven major clusters (Fig 2). The isolates Rs-1, Rs -3, Rs-2, Rs-4, Rs-5 and Rs-6 (UAS Dharwad, Saptapur, Garag, UAS Bangalore, Doddaballapur and Gubbi) were found in one cluster, isolates Rs-18, Rs-19, Rs-22 and Rs-23(Chintamani, Shimoga, Kolar and Chikkmangalur) formed second cluster, Rs-11 and Rs-12 of (Chalageri and Arabhavi) isolates were found in third cluster, isolates Rs-7 and Rs-9 (Hosalli and Doddaballapur) found in fourth cluster with high genetic similarity. Rs-15, Rs-17 and Rs-24 of Mysore, Chikkaballapur and Davangere isolates were found in fifth cluster. Isolates Rs-8, Rs-14, Rs-20, Rs-13 and Rs-16 isolates of Bannikoppa, Khanapur, Shimoga, Gokak and Hunsur were found in sixth cluster. Isolates of Rs-10 and Rs-21 (Ranebennur and Ramanagar) isolates were found in seventh cluster.

Table 8. Banding profile of different primers for different isolates of Ralstonia solanacearum

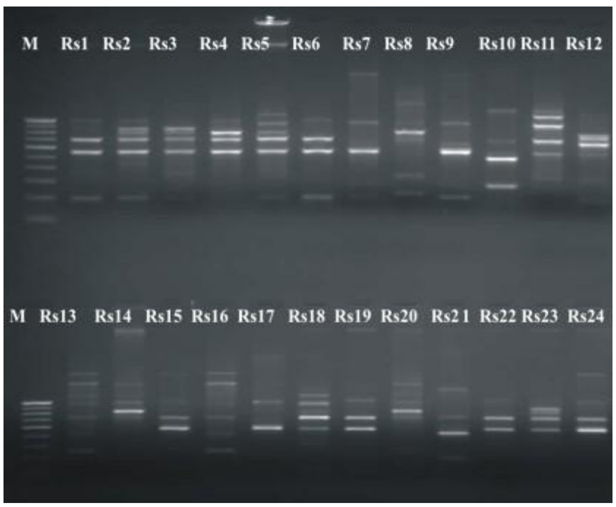
Primers	Total bands	Polymorphic band	% Polymorphism
OPA 1	8	7	87.5
OPA2	9	7	77.77
OPA3	4	3	75.00
OPA4	6	5	83.33
OPA5	8	5	62.50
OPA6	9	7	77.77
OPA7	7	6	85.71
OPA8	11	9	81.81
OPA9	5	4	80.00
OPA10	9	9	100.0
OPB1	6	4	66.66
OPB2	8	7	87.5
OPB3	6	6	100.0
OPB4	12	11	91.66
OPB5	6	5	83.33
OPB6	7	5	71.42
OPB7	9	5	55.55
OPB8	9	6	66.66
OPB9	8	5	62.50
OPB10	4	2	50.00
OPF1	12	12	100.0
OPF2	13	7	53.84
OPF3	16	6	37.50
OPF4	12	8	66.66
OPF5	5	4	80.00
OPF 6	6	3	50.00
OPF7	6	6	100
OPF8	8	8	100
OPF9	5	3	60.00
OPF10	7	4	57.14
Total	241	139	73.93

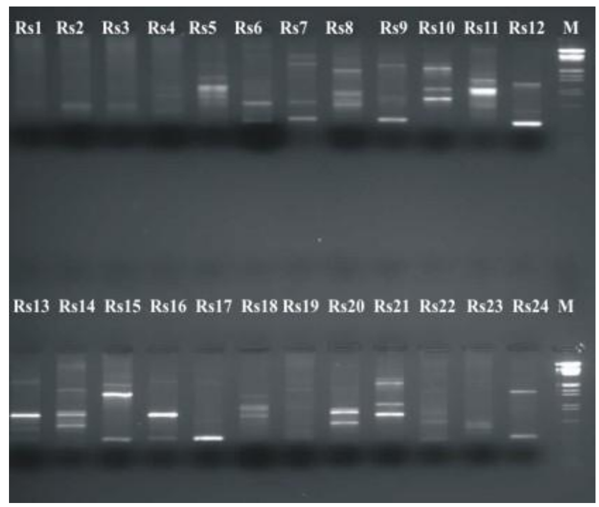




OPA 1

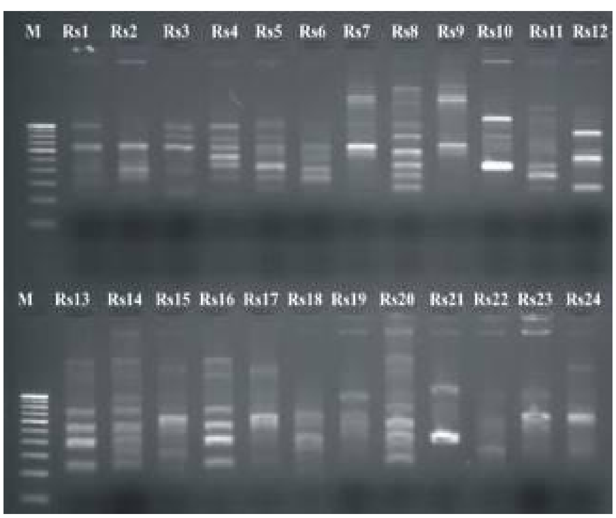
OPA 6

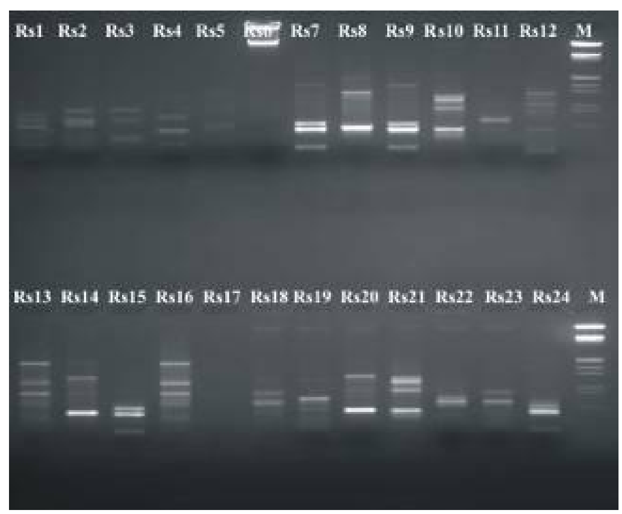




OPA 2

OPA 8





OPA 4

OPA 7

Plate 11. **RAPD banding pattern in *Ralstonia solanacearum* isolates**

Table 9. Similarity coefficient of twenty four isolates of Ralstonia solanacearum

Isolates	Rs-1	Rs-2	Rs-3	Rs-4	Rs-5	Rs-6	Rs-7	Rs-8	Rs-9	Rs-10	Rs-11	Rs-12	Rs-13	Rs-14	Rs-15	Rs-16	Rs-17	Rs-18	Rs-19	Rs-20	Rs-21	Rs-22	Rs-23	Rs-24
Rs-1	1.00																							
Rs-2	0.52	1.00																						
Rs-3	0.53	0.51	1.00																					
Rs-4	0.47	0.47	0.49	1.00																				
Rs-5	0.44	0.44	0.49	0.46	1.00																			
Rs-6	0.43	0.40	0.43	0.36	0.50	1.00																		
Rs-7	0.24	0.21	0.23	0.22	0.30	0.31	1.00																	
Rs-8	0.20	0.23	0.31	0.28	0.38	0.35	0.37	1.00																
Rs-9	0.29	0.21	0.27	0.25	0.23	0.30	0.61	0.31	1.00															
Rs-10	0.26	0.19	0.29	0.24	0.35	0.26	0.23	0.32	0.27	1.00														
Rs-11	0.25	0.24	0.28	0.26	0.43	0.35	0.25	0.25	0.29	0.29	1.00													
Rs-12	0.28	0.28	0.35	0.29	0.32	0.38	0.27	0.32	0.26	0.28	0.36	1.00												
Rs-13	0.29	0.28	0.32	0.33	0.37	0.36	0.32	0.42	0.29	0.28	0.23	0.30	1.00											
Rs-14	0.29	0.21	0.23	0.24	0.38	0.42	0.38	0.46	0.29	0.33	0.27	0.31	0.44	1.00										
Rs-15	0.25	0.26	0.28	0.21	0.34	0.22	0.33	0.29	0.33	0.29	0.31	0.27	0.31	0.25	1.00									
Rs-16	0.29	0.27	0.32	0.24	0.37	0.35	0.30	0.42	0.32	0.29	0.26	0.30	0.59	0.47	0.35	1.00								
Rs-17	0.22	0.23	0.27	0.28	0.36	0.36	0.35	0.29	0.35	0.29	0.29	0.27	0.32	0.31	0.51	0.36	1.00							
Rs-18	0.27	0.26	0.30	0.26	0.33	0.36	0.25	0.36	0.28	0.29	0.35	0.31	0.39	0.34	0.27	0.41	0.24	1.00						
Rs-19	0.39	0.32	0.36	0.33	0.38	0.37	0.25	0.25	0.25	0.28	0.34	0.33	0.30	0.31	0.29	0.32	0.26	0.42	1.00					
Rs-20	0.32	0.29	0.29	0.24	0.40	0.36	0.44	0.47	0.41	0.39	0.28	0.34	0.44	0.49	0.35	0.50	0.37	0.37	0.29	1.00				
Rs-21	0.29	0.20	0.26	0.23	0.34	0.38	0.36	0.43	0.32	0.38	0.30	0.32	0.33	0.37	0.28	0.32	0.20	0.39	0.39	0.38	1.00			
Rs-22	0.34	0.19	0.33	0.25	0.33	0.36	0.26	0.26	0.20	0.31	0.30	0.30	0.29	0.29	0.26	0.35	0.27	0.39	0.45	0.35	0.32	1.00		
Rs-23	0.38	0.21	0.39	0.33	0.37	0.39	0.30	0.32	0.22	0.26	0.25	0.35	0.25	0.32	0.22	0.26	0.23	0.33	0.36	0.34	0.31	0.43	1.00	
Rs-24	0.33	0.33	0.39	0.28	0.41	0.39	0.35	0.33	0.33	0.28	0.31	0.31	0.32	0.33	0.42	0.39	0.43	0.36	0.37	0.43	0.35	0.42	0.34	1.00

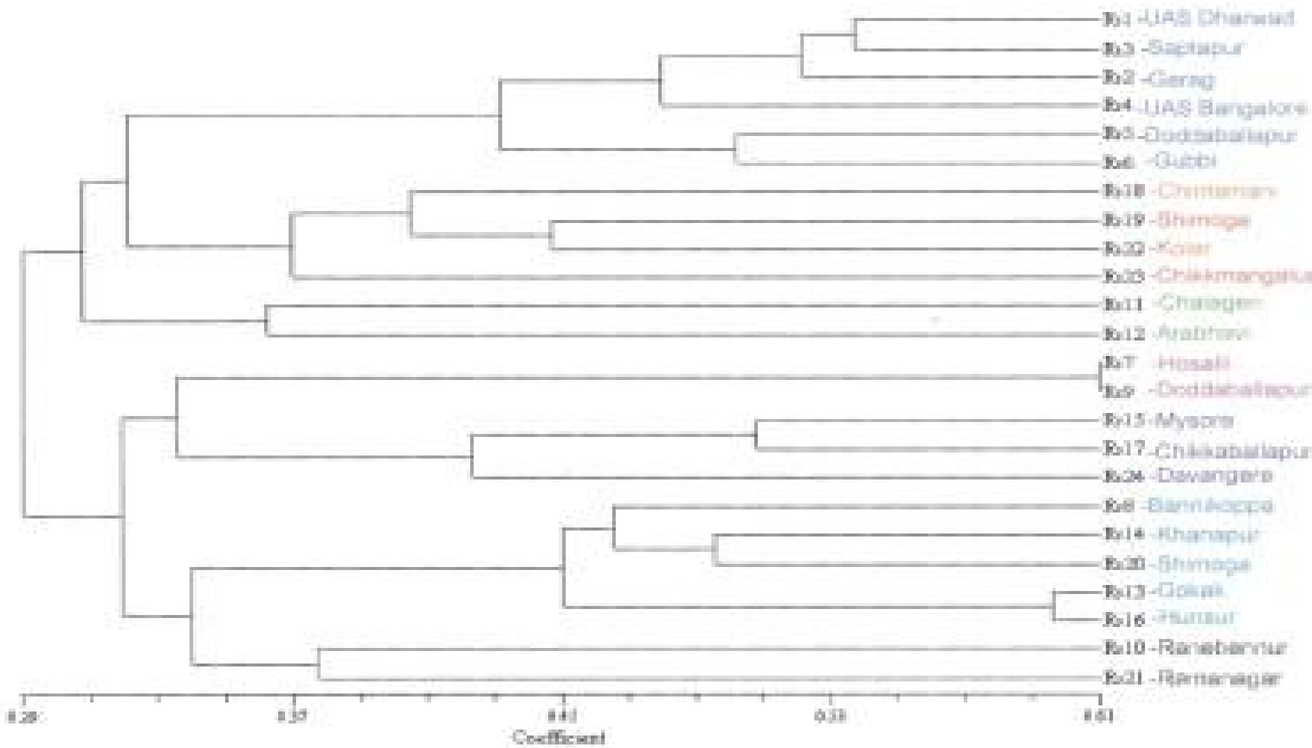


Fig. 2. Dendrogram for twenty three isolates of R. solanacearum based on RAPD analysis

Fig 2. Dendrogram for twenty three isolates of R. solanacearum based on RAPD analysis

4.6.3 Detection of Meloidogyne incognita by 18s rDNA gene amplification

The cultures of M. incognita (UAS Dharwad, Saidapur, Arabhavi and UAS Bangalore) were detected by 18s small subunit ribosomal gene using two sets of primers viz., 18s Forward primer (5′-ATGTATAAGTTTAATCGTTTTAACGA -3′) and 18s Reverse primer (5′-GTATGTACCAACTATTTAGTAGGT- 3′) and two primers specially designed for this study. The positions and sequences of primers are given in Fig 3. The predicted length of product is 1300 bp. The PCR performed with the specific primer combination produced only the single expected fragment of 1.3 kbp for all isolates of M. incognita. However no PCR amplified products were obtained in the negative control lacking DNA template (Plate 12).

4.7 Serodiagnosis

4.7.1 Serological detection of Fusarium oxysporum. f. sp. lycopersici

Antiserum against F. o. f. sp. lycopersici was prepared using 3-month old female albino rabbit by giving four (weekly) intramuscular injections with purified preparation of the fungus suspended in PBS at concentration of 1mg/ml as described in “Material and Methods”.

4.7.1.1 Determination of titre of antiserum by tube precipitation test

After production and purification of antisera from F. o. f. sp. lycopersici antigen, they were tested for titre value by tube precipitation test. The highest dilution at which reaction of antigen and antibody could be demonstrated was considered the titre of the serum. It was evident from Table 10, that the sera possessed a reaction up to 1:1280 dilutions.

4.7.1.2 Titre of polyclonal antibody against Fusarium oxysporum. f. sp. lycopersici antigen determined by DAC and DAS- Elisa techniques

Titre of antisera was determined by two-fold serial dilutions of antiserum from 100 to 204800 (Table 11, Plate 13 and Fig 4). Purified antigen of F. o. f. sp. lycopersici used as an antigen. The titre of antisera against antigen was determined by DAS - ELISA, it was high enough to be detectable up to 51200 dilution, while the DAC- ELISA technique detected only up to 25600 dilution. This was found out based on colour changes in the substrates at optical density value of 405 nm wave length, using ELISA reader.

4.7.1.3 Reactivity antiserum to the purified antigen of Fusarium oxysporum. f.sp. lycopersici

Reactivity of F. o. f. sp. lycopersici antiserum to the purified antigen of F. o. f. sp. lycopersici was determined using DAC - ELISA. The antigen was diluted with phosphate buffer to adjust the given concentration 0.1 to 204.8 µg. The result showed that the antigens at concentrations of 3.2 µg (and above) were detected using DAC -ELISA (Table 12, Fig 5 and Plate 14). In contrast, antigens of 0.1 to 1.6 µg were not detected and gave negative result.

4.7.1.4 Sensitivity of antisera to the threshold concentration of conidia of Fusarium oxysporum. f. sp. lycopersici determined by DAC-ELISA

Sensitivity of antiserum was also determined, the plates were coated with conidia of F. o. f. sp. lycopersici, Serial ten-fold dilutions of conidial suspension from 10^{10} to 10^{0}/well were used. The polyclonal antibody had the highest level of reactivity to F. o. f. sp. lycopersici conidial antigen, Sensitivity of PAb was precise enough to detect spore concentrations as low as 50 conidia/well by DAC- ELISA technique (Plate 15).

4.7.1.5 Specificities Fusarium oxysporum. f. sp. lycopersici of PAb using the DAC -ELISA technique

The specificity of antiserum produced against F. o. f. sp. lycopersici was tested by DAC- ELISA technique. The twenty three isolates F. o. f. sp. lycopersici collected from different agroclimatic zones of Karnataka reacted positively (Table 13, Fig 6 and Plate 16). Among other fungi also evaluated to determine the possibility of non specific cross reactions, viz. Rhizoctonia solani, Sclerotium rolfsii, Alternaria alternata showed negative result against antisera of F. o. f. sp. lycopersici and antiserum cross-reacted with other Fusarium sp. (other than F. o. f. sp. lycopersici) wherein, very week ELISA values (negative response) were obtained.

Fig 3. 18s rDNA gene of Meloidogyne incognita and amplified position of primers

1.3 kb

M-1kb Marker

1- Dharwad (USA Dharwad)

2-Dharwad (Saidapur)

3-Belguam (Arabhavi)

4-Bangalore (USA- Bangalore)

C-Control

Plate 12. Detection of Meloidogyne incognita by 18S rDNA gene amplification

Table 10. Determination of titre of Fusarium oxysporum f. sp. lycopersici antiserum by tube precipitation test

Antiserum dilution	Reaction
10	++++
20	++++
40	++++
80	+++
160	+++
320	++
640	++
1280	+
2560	-
5120	-
10240	-
Negative control (PBS)	-

- : no precipitation
+ : slight precipitate
++ : moderate precipitate
+++ : heavy precipitate
+++ : very heavy precipitate

Table 11. Fusarium oxysporum f. sp. lycopersici antiserum titre (dilution end point) determined by ELISA techniques

Antiserum dilution	DAC- ELISA		DAS- ELISA	
	Visual observation	OD_{405nm}	Visual observation	OD_{405nm}
x100	+	0.791	+	0.882
200	+	0.732	+	0.856
400	+	0.698	+	0.741
800	+	0.564	+	0.699
1600	+	0.492	+	0.635
3200	+	0.324	+	0.591
6400	+	0.221	+	0.452
12800	+	0.213	+	0.358
25600	+	0.201	+	0.331
51200	-	0.098	+	0.255
102400	-	0.089	-	0.132
204800	-	0.084	-	0.096
Negative control (PBS)	-	0.083	-	0.089

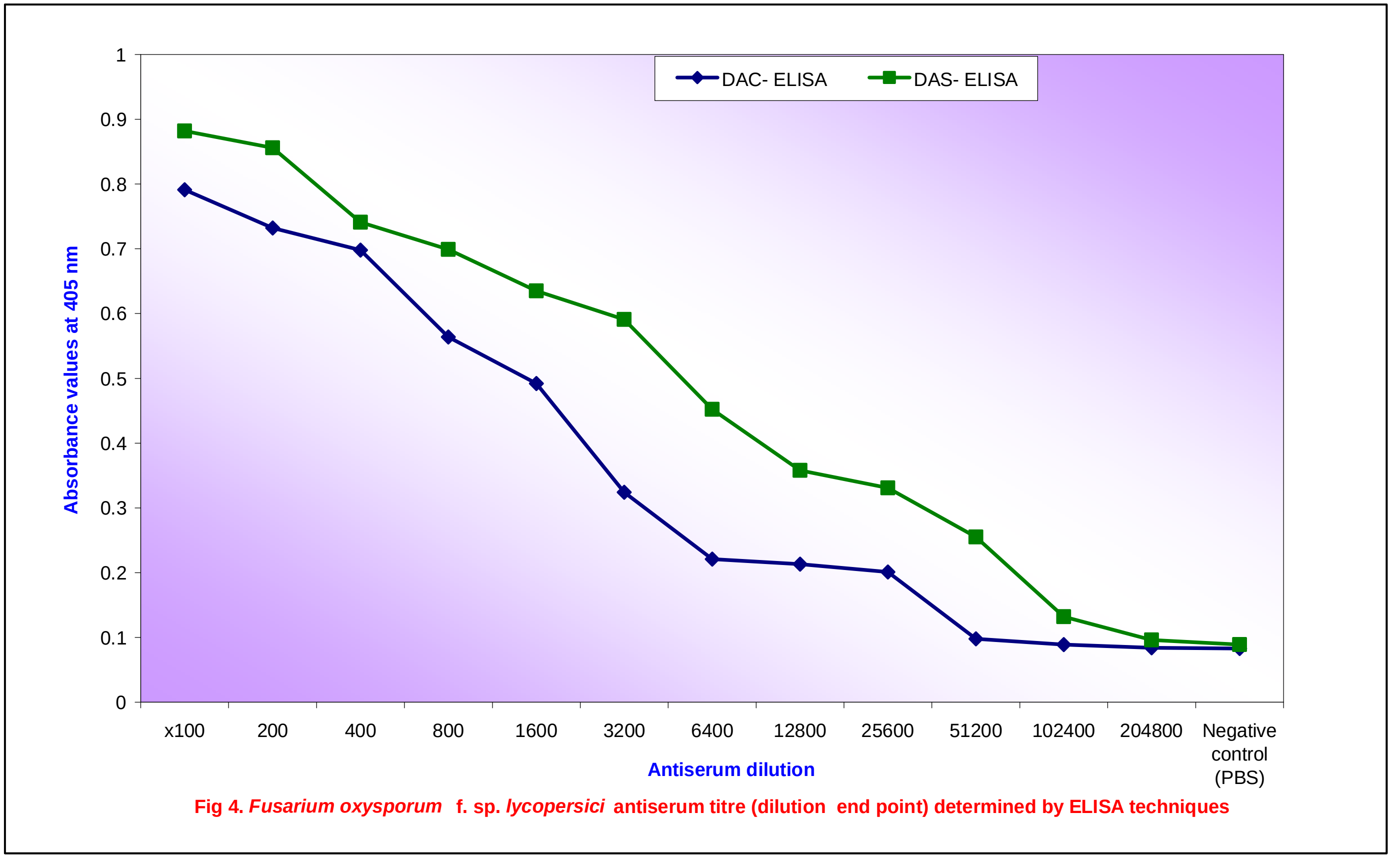


Fig 4. Fusarium oxysporum f. sp. lycopersici antiserum titre (dilution end point) determined by ELISA techniques

Table 12. Reactivity of Fusarium oxysporum f. sp. lycopersici antiserum and antigen determined by DAC- ELISA technique

Antigen concentration (µg)	Visual observation	OD_{405nm}
0.1	-	0.089
0.2	-	0.091
0.4	-	0.098
0.8	-	0.124
1.6	-	0.132
3.2	+	0.299
6.4	+	0.398
128	+	0.489
256	+	0.543
512	+	0.652
1024	+	0.725
2048	+	0.863
Negative control (PBS)	-	0.084

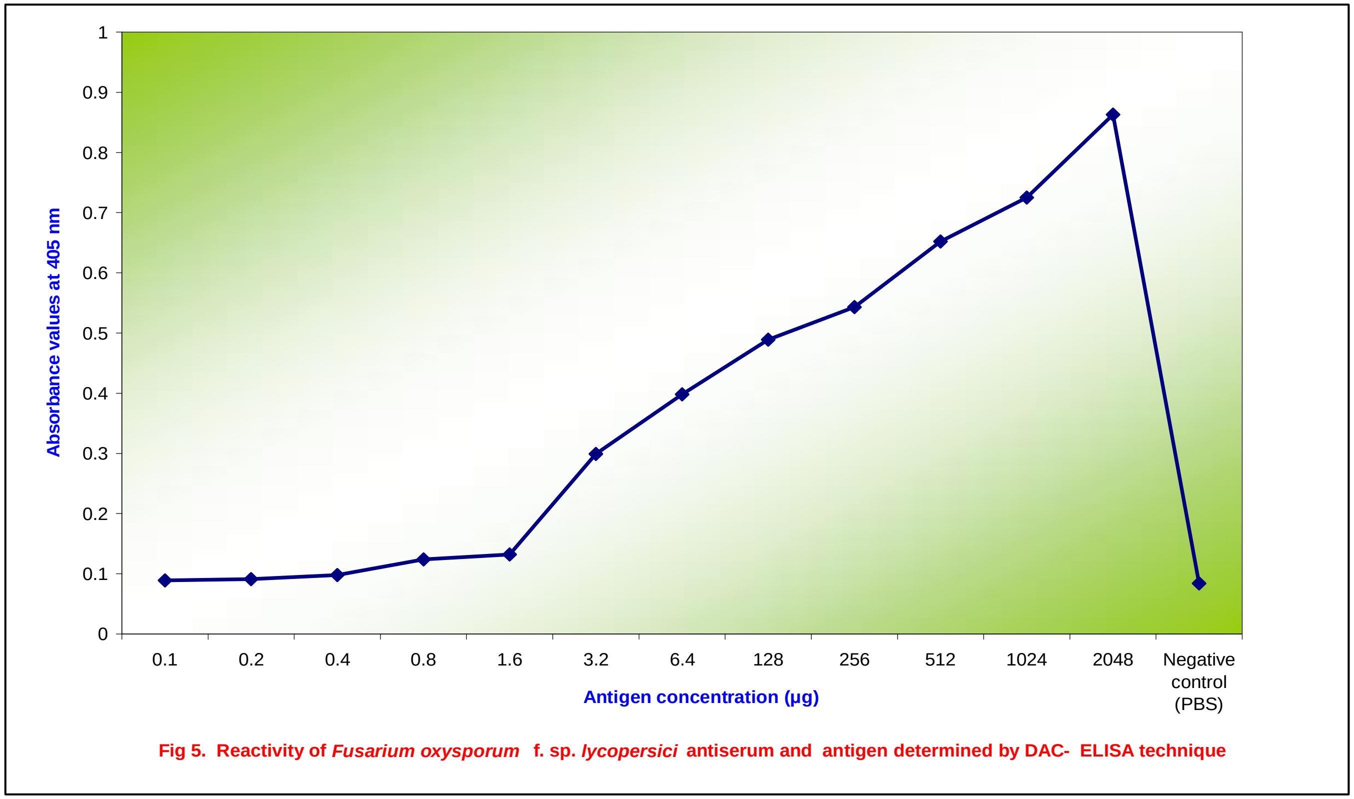
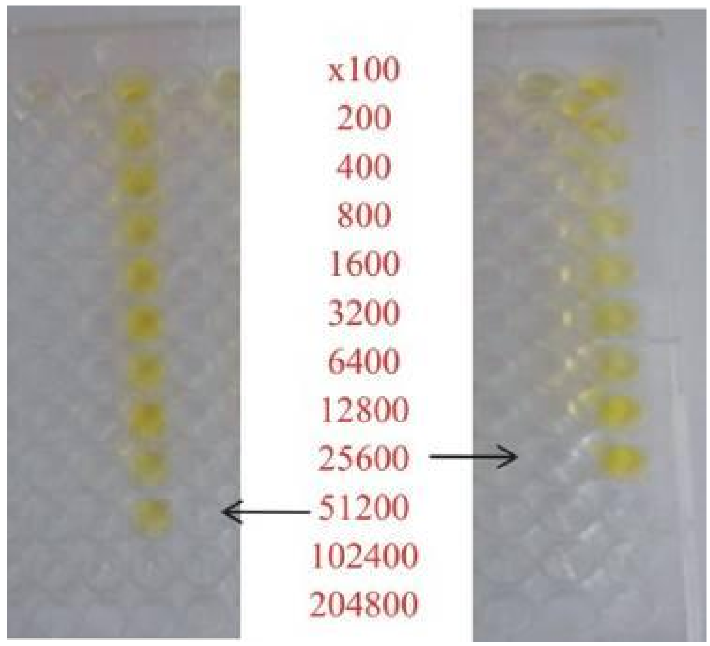


Fig 5. Reactivity of Fusarium oxysporum f. sp. lycopersici antiserum and antigen determined by DAC- ELISA technique



DAS- ELISA **DAC - ELISA**

Plate 13. Titre of polyclonal antibody against Fusarium oxysporum f. sp. lycopersici antigen determined by ELISA techniques

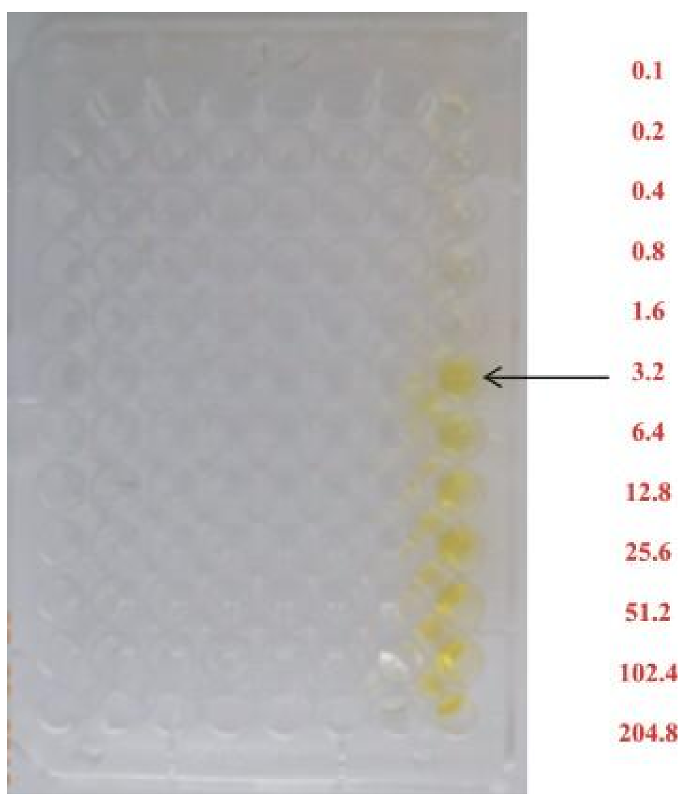


Plate 14. Reactivity of Fusarium oxysporum f. sp. lycopersici antiserum and antigen determined by DAC- ELISA technique

4.7.1.6 Sensitivities of the DAC-ELISA technique for the detection of Fusarium oxysporum. f. sp. lycopersici from plants and soils

The DAC- ELISA technique was used in the detection of F. o. f. sp. lycopersici from naturally infected soil and plant samples collected from field (Table 14 and Plate 17). The rhizosphere soil of tomato collected from different tomato growing areas of Karnataka (UAS Dharwad, Saidapur, Garag, Arabhavi, Khanapur, and Kolar) infested with Fusarium wilt gave positive result by DAC- ELISA- technique. Plant samples infected with F. o. f. sp. lycopersici collected from different locations also reacted with antisera of F. o. f. sp. lycopersici, where as healthy tomato plant samples did not react.

4.7.2 Serological detection of Ralstonia solanacearum

Polyclonal antibodies were developed in a 3-month old female albino rabbit, using virulent fluidal bacterial whole cells in the presence of Freund's incomplete adjuvant as described in the ‘Material and Methods’.

4.7. 2.1 Determination of titre of antiserum

Titer of antisera was determined by serial dilutions of antiserum from 10 to 10240. Highest dilution at which reaction of antigen and antibody could be demonstrated was considered the titre of the serum. It was evident from Table 15.That the sera possessed a highest titre of 1: 2560 dilution.

4.7.2.2 Effectiveness of ELISA techniques for detection of Ralstonia solanacearum

Results of testing on the effectiveness of ELISA techniques, i.e. DAC and DAS-ELISA with varying concentration of cells per ml i.e 10^{-8} to $10,^{-2}$ showed different sensitivities in detection (Table 16, Fig 7 and Plate 18).The DAS-ELISA technique was capable of detecting R. solanacearum up to10^{-3} cells/ml, while the DAC- ELISA technique detected only up to 10^{-5} cells/ml. This indication was based on colour changes in the substrates and optical density value at 405 nm wave length using ELISA reader. This indicates that the DAS- ELISA technique was more sensitive than DAC- ELISA technique.

4.7.2.3 Specificity of PAb for Ralstonia solanacearum using the DAC- ELISA technique

The PAbs produced using antigen of R. solanacearum showed specific reactions to R. solanaceaum of 24 isolates collected from different agro climatic zones of Karnataka (Table17, Fig 8 and Plate 19). And one other bacterial pathogen evaluated to check the cross reaction of antibody produced against R. solanacearum, only antigen of Xanthomonas sp. showed negative result using DAC- ELISA technique.

4.7.2.4 Testing sensitivities of the DAC- ELISA Technique for the detection of Ralstonia solanacearum from plants and soils samples

Results of testing the sensitivity of the DAC- ELISA technique using naturally infested soil and plant material collected from different regions of Karnataka are presented in Table 18 and Plate 20. Soil from tomato rhizosphere infested with R. solanacearum (UAS Dharwad, Garag, Khanapur, Gokak, UAS Bangalore, Kolar, Ramanagar, Arabhavi) showed positive result and soil collected from healthy tomato rhizosphere (UAS Dharwad) did not react.

The technique was also able to detect R. solanacearum directly from tomato plant samples infected with R. solanacearum collected from different location. Infected samples showed positive results and healthy plant sample of tomato did not show positive reaction. The high value of OD_{405} (0.603-0.725) on plant samples infected with R. solanacearum indicated that the population of R. solanacearum in the plant samples was high as compared to OD value of soil sample (0.468 to 0.668) collected from different location.

4.7.3 Serological detection of root knot nematode

Antisera were produced in female albino rabbit. Four weekly intramuscular injections with antigen preparations (homogenates of J_2 and saccate adult females, 1mg/ml) as described in ‘Material and Methods’.

Table 13. Specificities of Fusarium oxysporum f. sp. lycopersici PAb for detection of fungal antigens by DAC -ELISA technique

Fungal antigen	Visual Observation	OD_{405nm}
Fol-1	+	0.972
Fo1-2	+	0.707
Fo1-3	+	0.795
Fo1-4	+	0.985
Fo1-5	+	0.494
Fo1-6	+	0.711
Fo1-7	+	0.992
Fo1-8	+	0.446
Fo1-9	+	0.625
Fo1-10	+	0.934
Fo1-11	+	0.824
Fo1-12	+	0.698
Fo1-13	+	0.591
Fo1-14	+	0.793
Fo1-15	+	0.775
Fo1-16	+	0.828
Fo1-17	+	0.860
Fo1-18	+	0.896
Fo1-19	+	0.778
Fo1-20	+	0.840
Fo1-21	+	0.920
Fo1-22	+	0.912
Fo1-23	+	0.658
Rhizoctonia solani	-	0.174
Sclerotium rolfsii	-	0.155
Alternaria alternata	-	0.165
Fusarium sp.	+	0.184
Fusarium sp.	+	0.165
Fusarium sp	+	0.193
Control (PBS)	-	0.075

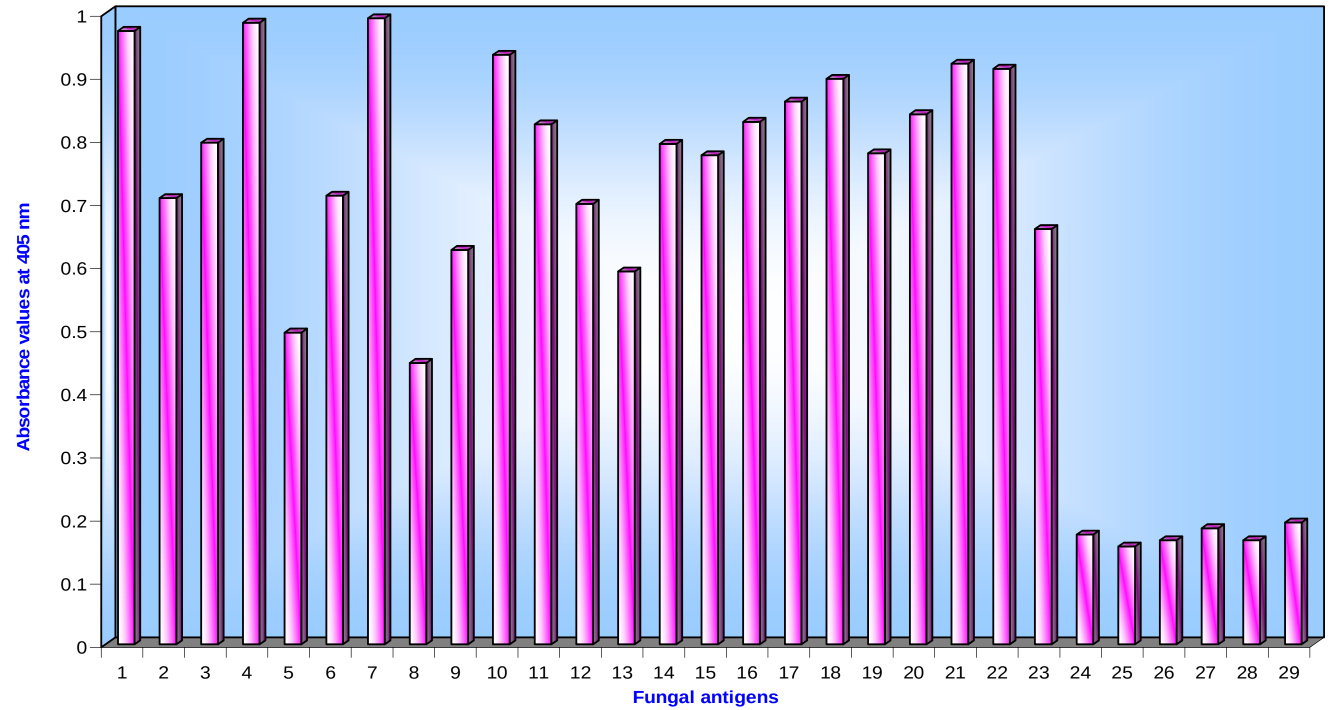


Fig 6. Specificities of *Fusarium oxysporum* f. sp. *lycopersici* PAb for detection of fungal antigens

Fig 6. Specificities of Fusarium oxysporum f. sp. lycopersici PAb for detection of fungal antigens

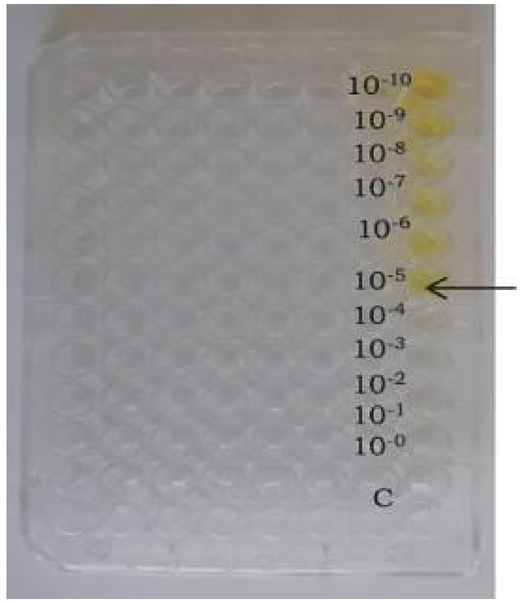
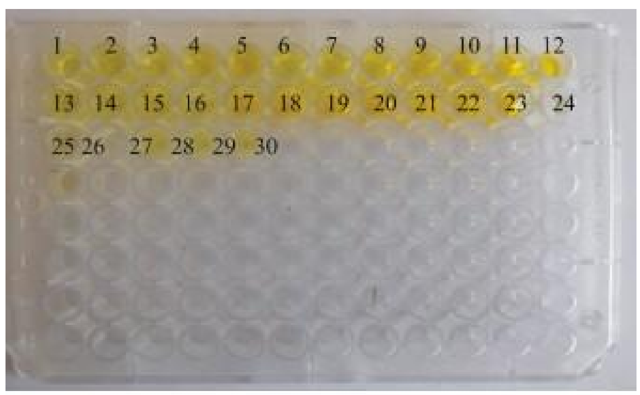


Plate 15. Sensitivity of antisera to the threshold conidial concentration of Fusarium oxysporum f. sp. lycopersici by DAC-ELISA.

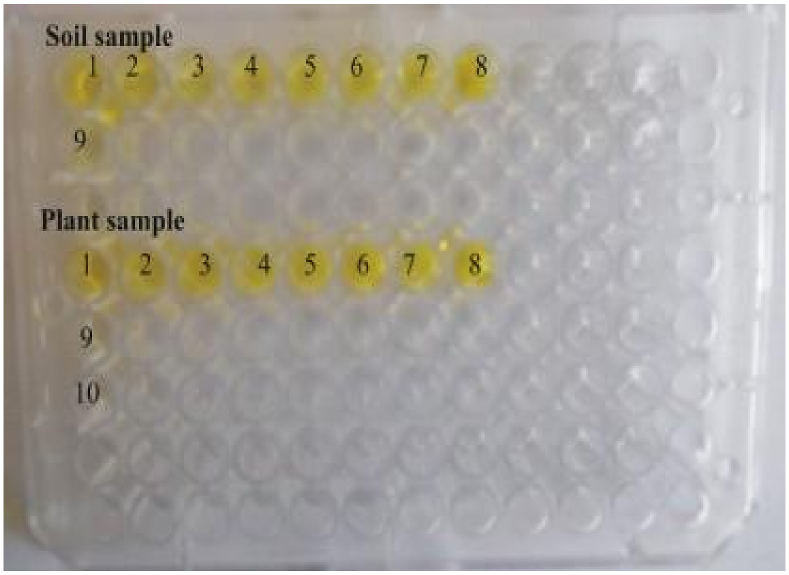


1. Fol-1
2.Fo1-2
3. Fo1-3
4.Fo1-4
5.Fo1-5
6.Fo1-6
7.Fo1-7
8.Fo1-8
9.Fo1-9
10.Fo1-10
11.Fo1-11
12.Fo1-12
13.Fo1-13
14.Fo1-14
15.Fo1-15
16.Fo1-16
17.Fo1-17
18.Fo1-18
19.Fo1-19
20.Fo1-20
21.Fo1-21
22.Fo1-22
23.Fo1-23
24.*Rhizoctonia solani*
25.*Sclerotium rolfsii*
26.*Alternaria alternata*
27.*Fusarium* sp.
28.*Fusarium* sp.
29.*Fusarium* sp
30.Negative control (PBS)

Plate 16. Specificities Fusarium oxysporum f. sp. lycopersici PAb for detection of fungal antigens by using DAC -ELISA technique

Table 14. Sensitivity of the DAC- ELISA technique for the detection of Fusarium oxysporum f. sp. lycopersici from wilt infested soil and plant samples collected from field

Soil or Plant Sample	Locality	Visual observation	OD_{405nm}
Soil from tomato rhizosphere infested with F.o.f. sp. lycopersici	UAS Dharwad	+	0.542
	Saidapur	+	0.468
	Garag	+	0.586
	Arabhavi	+	0.620
	Khanapur	+	0.507
	Ramanagar	+	0.432
	Kolar	+	0.492
	Shimoga	+	0.582
Soil from healthy tomato rhizosphere	UAS Dharwad	-	0.145
Tomato plant infected with F.o. f. sp. lycopersici	Saidapur	+	0.756
	Garag	+	0.672
	Arabhavi	+	0.568
	Khanapur	+	0.678
	Ramanagar	+	0.778
	Kolar	+	0.589
	Shimoga	+	0.541
Healthy tomato plant	UAS Dharwad	-	0.182
Negative control (PBS)		-	0.124



Soil sample	**Plant sample**
1.UAS Dharwad	1.UAS Dharwad
2. Saidapur	2.Saidapur
3. Garag	3. Garag
4. Arabhavi	4.Arabhavi
5. Khanapur	5. Khanapur
6.Ramanagar	6.Ramanagar
7. Kolar	7. Kolar
8. Shimoga	8. Shimoga
9.UAS Dharwad	9.UAS Dharwad
	10. Negative contro (PBC)

Plate 17. Detection of Fusarium oxysporum f. sp. lycopersici from plant and soil samples by DAC- ELISA technique

Table 15. Determination of titre of Ralstonia solanacearum antiserum by tube precipitation test

Antiserum dilution	Reaction
10	++++
20	++++
40	+++
80	+++
160	+++
320	++
640	++
1280	++
2560	+
5120	-
10240	-
Negative control (PBS)	-

- : no precipitation
+ : slight precipitate
++ : moderate precipitate
+++ : heavy precipitate
+++ : very heavy precipitate

Table 16. Sensitivity of ELISA techniques based on Visual observation results and Absorbent value (OD_{405nm})

Dilutions (Cfu/ ml)	ELISA Techniques			
	DAC- ELISA		DAS-ELISA	
	Visual observation	OD_{405nm}	Visual observation	OD_{405nm}
10^8	+	0.652	+	0.984
10^7	+	0.528	+	0.725
10^6	+	0.346	+	0.613
10^5	+	0.299	+	0.421
10^4	-	0.121	+	0.314
10^3	-	0.098	+	0.211
10^2	-	0.087	-	0.134
Negative control (PBS)		0.85		0.089

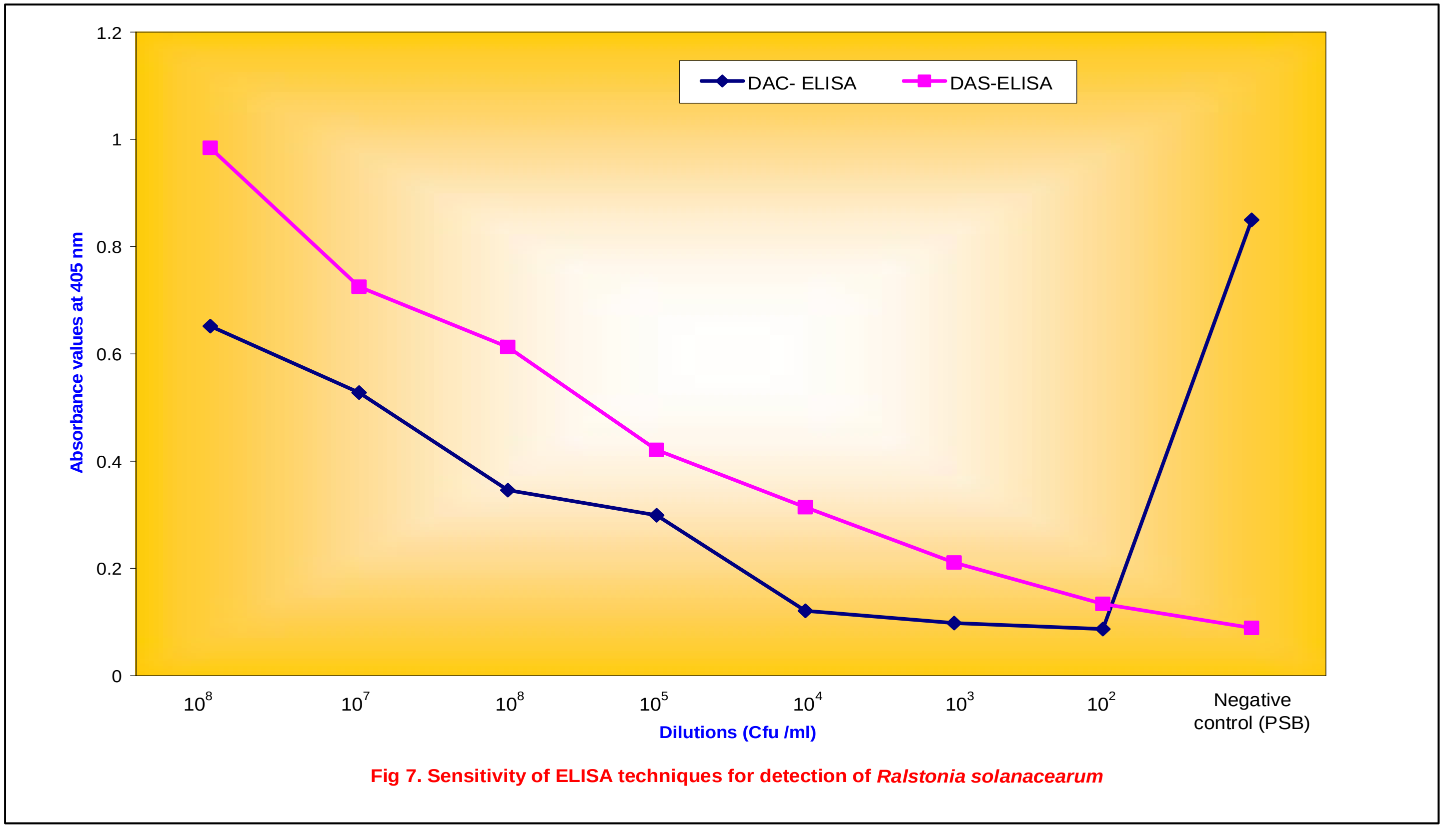


Fig 7. Sensitivity of ELISA techniques for detection of Ralstonia solanacearum

Table 17. Specificities of Ralstonia solanacearum PAb for detection of bacterial antigens by DAC-ELISA technique

Bacterial antigens	Visual observation	OD_{405nm}
Rs-1	+	0.936
Rs-2	+	0.841
Rs-3	+	0.652
Rs-4	+	0.859
Rs-5	+	0.789
Rs-6	+	0.891
Rs-7	+	0.881
Rs-8	+	0.684
Rs-9	+	0.632
Rs-10	+	0.973
Rs-11	+	0.860
Rs-12	+	0.658
Rs-13	+	0.776
Rs-14	+	0.625
Rs-15	+	0.691
Rs-16	+	0.932
Rs-17	+	0.801
Rs-18	+	0.811
Rs-19	+	0.784
Rs-20	+	0.665
Rs-21	+	0.743
Rs-22	+	0.756
Rs-23	+	0.811
Rs-24	+	0.853
Xanthomonas spp.	-	0.125
Negative control (PBS)	-	0.089

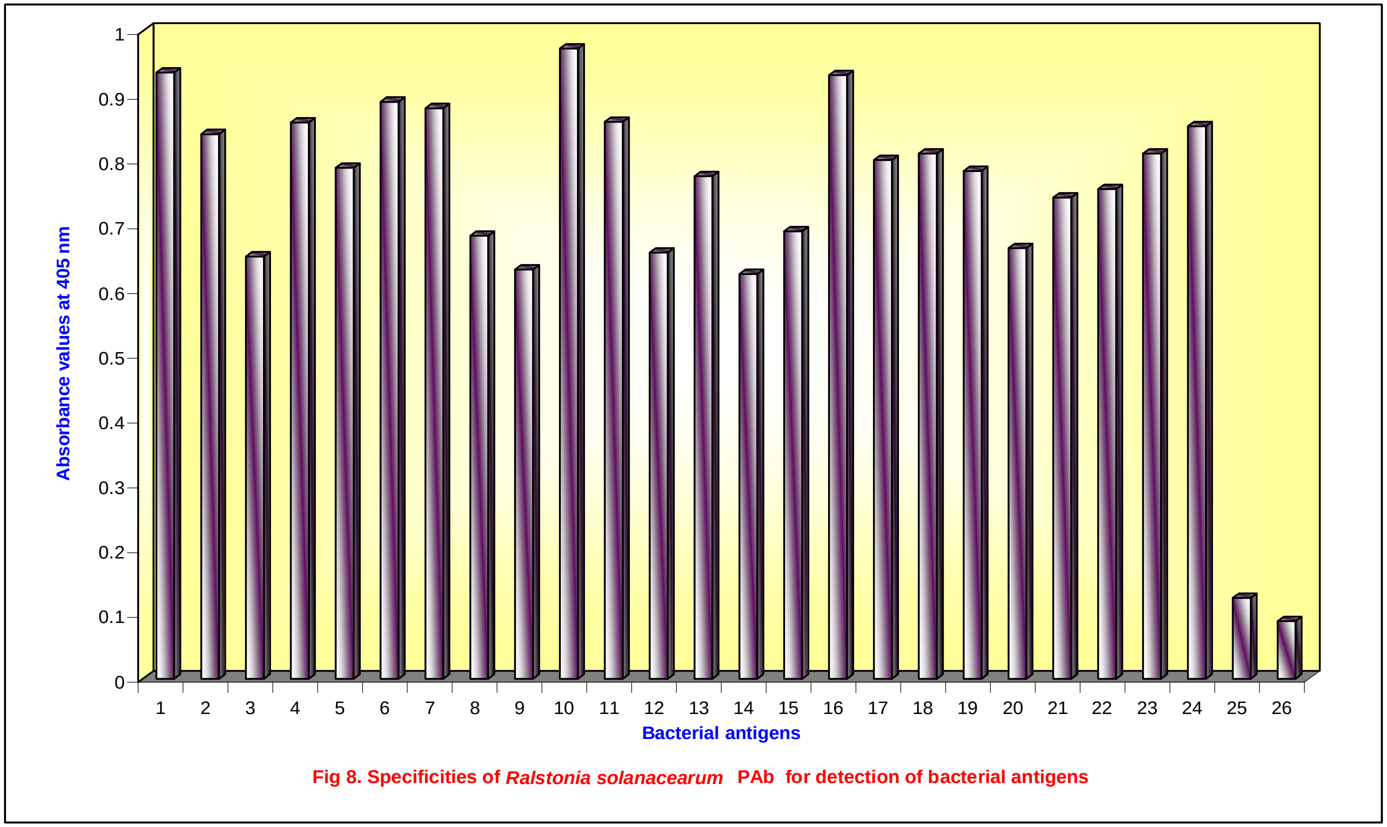


Fig 8. Specificities of Ralstonia solanacearum PAb for detection of bacterial antigens

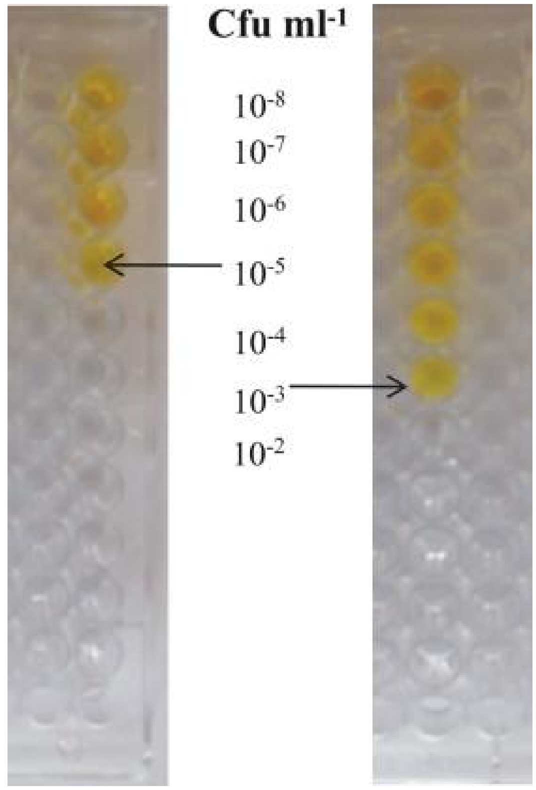
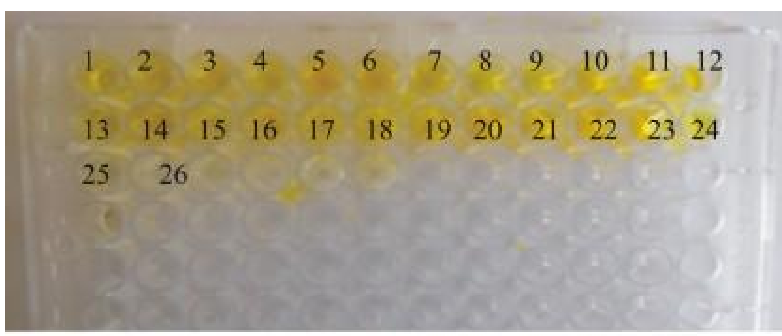


Plate 18. Effectiveness of ELISA techniques for detection of Ralstonia solanacarum

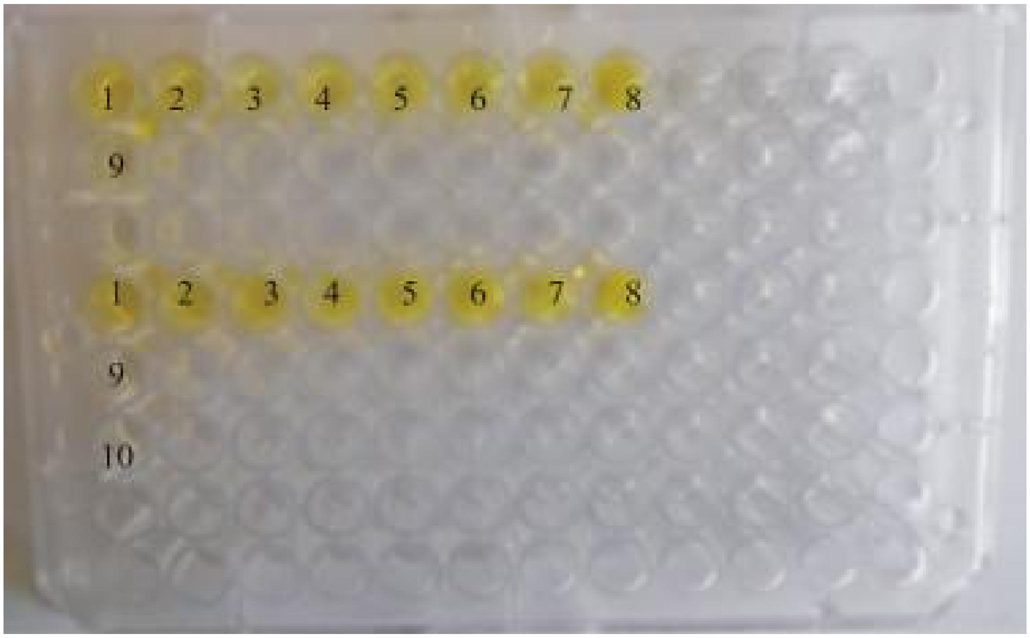


1.Rs1	14.Rs14
2.Rs2	15.Rs15
3.Rs3	16.Rs16
4.Rs4	17.Rs17
5.Rs5	18.Rs18
6.Rs6	19.Rs19
7.Rs7	20.Rs20
8.Rs8	21.Rs21
9.Rs9	22.Rs22
10.Rs10	23.Rs23
11.Rs11	24.Rs24
12.Rs12	25. *Xanthomonas* sp.
13.Rs13	26. Negative control (PBS)

Plate 19. Specificities of Ralsonia solanacearum PAb for detection of bacterial antigen using DAC- ELISA technique

Table 18. Sensitivity of the DAC- ELISA technique for the detection of Ralstonia solanacearum from wilt infested soil and plant samples collected from field

Soil or plant sample	Locality	Visual observation	OD_{405nm}
Soil from tomato rhizosphere infested with R.solanacearum	UAS Dharwad	+	0.668
	Garag	+	0.542
	Khanapur	+	0.468
	Gokak	+	0.632
	UAS Bangalore	+	0.521
	Kolar	+	0.612
	Ramanagar	+	0.481
	Arabhavi	+	0.524
Soil from healthy tomato rhizosphere	UAS Dharwad	-	0.132
Tomato plant infected with R.solanaccurum	UAS, Dharwad	+	0.634
	Garag	+	0.714
	Khanapur	+	0.672
	Gokak	+	0.661
	UAS Bangalore	+	0.725
	Kolar	+	0.622
	Ramanagar	+	0.703
	Arabhavi	+	0.603
Healthy tomato plant	UAS Dharwad	-	0.135
Negative control (PBS)	-	-	0.093



1. UAS Dharwad
2. Garag
3. Khanapur
4. Gokak
5. UAS Bangalore
6. Kolar
7. Ramanagar
8. Arabhavi
9. UAS Dharwad

1. UAS Dharwad
2. Garag
3. Khanpur
4. Gokak
5. UAS Bangalore
6. Kolar
7. Ramanagar
8. Arabhavi
9. UAS Dharwad
10. Negative control (PBS)

Plate 20. Detection of Ralstonia solanacearum from plants and soil samples by DAC- ELISA technique

4.7.3.1 Sensitivity of the DAC- ELISA technique for the detection of root knot nematode

The antisera raised against root knot nematode (M. incognita) were tested against M. incognita antigen (homogenates of J2 and female antigen) using DAC- ELISA assay. All the four isolates collected from different regions (UAS Dharwad, Saidapur, Arabhavi and UAS Bangalore) showed positive reaction (Table 19, Fig 9 and Plate 21).

Result of testing the sensitivity of the DAC -ELISA technique using naturally infested soil samples of (UAS Dharwad, Saidapur, Arabhavi and UAS Bangalore) also showed positive reaction (Table 20 and Plate 22).

Table 19. Sensitivity of the DAC- ELISA technique for the detection of Meloidogyne incognita (homogenates of J2 and female antigen)

Locality	Visual observation	OD_{405nm}
UAS Dharwad	+	0.514
Saidapur	+	0.524
Arabhavi	+	0.498
UAS Bangalore	+	0.532
Negative control (PBS)	-	0.084

Table 20. Sensitivity of the DAC- ELISA technique for the detection of Meloidogyne incognita from soil samples collected from field

Locality	Visual observation	OD_{405nm}
UAS Dharwad	+	0.521
Saidapur	+	0.642
Garag	-	0.086
Arabhavi	+	0.593
Kolar	-	0.120
Ramanagar	-	0.123
UAS Bangalore	+	0.122
Khanapur	-	0.089
Gokak	-	0.088
Chikkballapur	-	0.090
Tumkur	-	0.132
Hosur	-	0.124
Negative control (PBS)	-	0.084

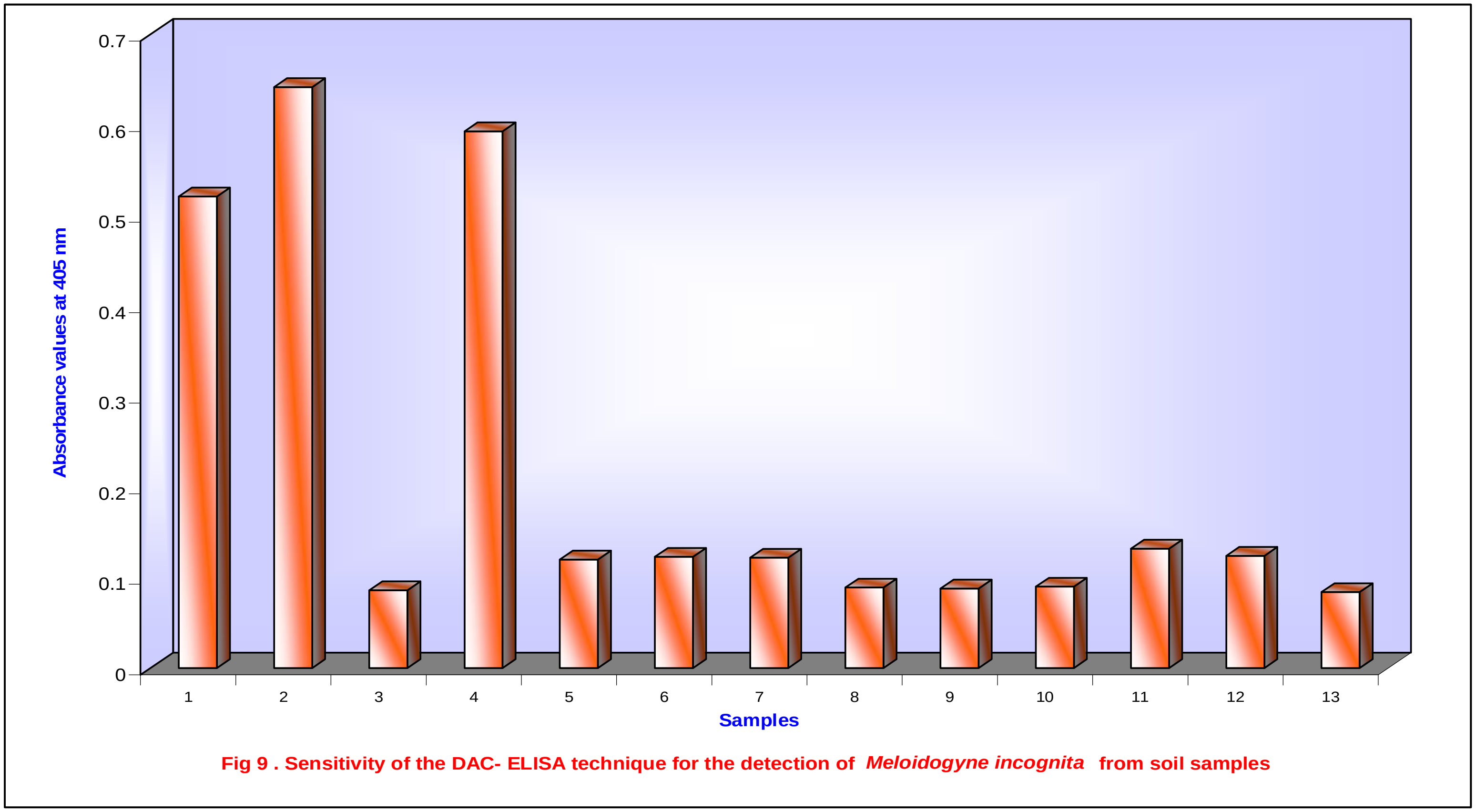


Fig 9 . Sensitivity of the DAC- ELISA technique for the detection of Meloidogyne incognita from soil samples

1.Dharwad (UAS Dharwad)
2. Dharwad (Saidapur)
3. Belgaum (Arabhavi)
4.Bangalore (UAS Bangalore)
5. Negative control (PBS)

Plate 21. Serological detection of Meloidogyne incognita by DAC- ELISA technique

1. UAS Dharwad
2. Saidapur
3. Garag
4. Arabhavi
5. Kolar
6. Ramanagar
7. UAS Bangalore
8. Khanapur
9. Gokak
10. Chikkballapur
11. Tumkur
12. Hosur
13. Negative control (PBS)

Plate 22. Sensitivity of the DAC- ELISA technique for the detection of Meloidogyne incognita from soil samples

5. DISCUSSION

Cultivated tomato (Solanum lycopersicum Mill.) is one of the world's most important crops due to high value of its fruits both for fresh market consumption and in numerous types of processed products (Giovanni et al., 2003). The volume of world production has increased approximately 10 percent since 1985, reflecting a substantial increase in dietary use of the tomato. One of the main constraints to tomato cultivation is damage caused by various soil borne pathogens (fungal, bacterial and nematode) which cause severe losses in production (Barone and Frusciante, 2007). Tomato is best adapted to warm and dry environments, but during the hot-wet season, yields are low due to poor fruit-setting caused by the high temperatures, as well as many severe disease problems. Among diseases, Fusarium wilt, caused by Fusarium oxysporum f. sp. lycopersici, is one of the important diseases on tomato world wide (Booth, 1971; Jones et al., 1991). Today, it has an extensive presence in all continents (Menzies and Jarvis, 1994; Brayford, 1996). The disease is further aggravated in presence of a plant parasitic nematode Meloidogyne incognita (Kofoid and White); In fact Meloidogyne speceies are widespread in many parts of the world and cause independently substantial yield losses, mainly in tropical and subtropical areas. Another vascular wilt caused by a bacterium Ralstonia solanacearum is also one of the most important diseases in tomato crops. Bacterial wilt is usually the most damaging, it has been reported that the incidence in tomato crops ranges 10 to 100 per cent incidence during summer (Jaw-Fen Wang, 2005).

The range of techniques available for identification and monitoring of soil-borne pathogens has expanded over past decades including immunological assays. Most recently, nucleic acid hybridization methods have become promising technique for diagnosis of soil borne diseases. The, results obtained during investigations into use of molecular and serological diagnosis of the three vascular pathogens affecting tomato are discussed here under.

5.1 Symptomatology

The fusarial wilt of tomato is caused by F. o. f. sp. lycopersici. The first indication of this disease is yellowing and drooping of the lower leaves. This symptom often occurs on one side of the plant or on one shoot. As the disease progresses growth is typically stunted, and little or no fruit develops. The browning of the vascular system is characteristic of the disease and generally can be used for its identification. Similar symptoms was also reported by earlier workers (Davis, 1982; Beckman, 1987 and Duniway, 1971).

Bacterial wilt of tomato is caused by R. solanacearum. The first visible symptoms of the disease was young leaves loosing their turgidity shown as drooping of leaves followed by the complete plant wilting within a few days. Recently wilted plants are green; this is a distinct symptom compared to other vascular diseases like Fusarium wilt, which develop yellowing of leaves. After wilting, a vascular discoloration could be observed. A cross section of stem of the plant with bacterial wilt produced a white, milky strand of bacterial cells in clear water. This ooze distinguishes the wilt caused by the bacterium from that caused by fungal pathogens. These symptoms was also observed by with Janse et al. (2004).

Meloidogyne incognita infected tomato plants were stunted in their growth with yellow coloured leaves. When infected plants were uprooted, deformed roots with prominent galls of varying size were noticed. Microscopic examination of the galls revealed the presence of egg, juveniles and females of M. incognita in the vascular region of roots. Similar types of obseravations were made by Taylor (1971) on tomato plant.

5.2 Collection and isolation of vascular pathogens affecting tomato

Vascular pathogens, viz. F. o. f. sp lycopersici, R. solanacearum and root knot nematode affected tomato plant samples were collected from different locations of Karnataka.

Standard tissue isolation method resulted in the pure culture of F. o. f. sp. lycopersici, further purified isolates were obtained by hyphal tip method. Similarly, Sachidananda (2005), Shyla (1998), Boby and Bhagyaraj (2003) isolated Fusarium by hyphal tip method.

The bacterium, R. solanacearum was isolated from the bacterial ooze obtained from the infected vascular tissue of the root portion by serial dilution method on sucrose peptone agar medium. The method described by Kelman (1954) was found to be ideal.

Different cultures of root knot nematode was established from single egg masses (Fargette et al., 1996) and have been maintained for several days on susceptible tomato Plants (Pusa ruby) in glasshouse cultures was as described by earlier scientist Blok et al.,1997.

5.3 Identification of pathogens

Study on morphological characters of F. o. f. sp. lycopersici isolates indicated that, mycelia of pathogen were cottony white to pink. Microconidia were abundant, oval-ellipsoid, straight to curved; macroconidia, sparse to abundant, three to five septate, fusoid-subulate and pointed at both ends and had pedicellate base. Three septate spores were predominant. Chlamydospores were both smooth and rough walled. The present studies is in agreement with Gerlach and Nirenberg (1982) who found that F. o. f. sp lycopersici was identified based on their morphological characters.

Studies on cultural and morphological features of 23 isolates of F. o. f. sp lycopersici was carried on pototo dextrose agar and these isolates were classified into three groups. All isolates showed moderate to abundant aerial mycelium which were initially white, cottony and fluffy. They later turned to pale pink colour. Chlamydospores terminal or intercalary, colony diameter ranged from 60.0- to 90.0 mm. sporulation varied from moderate to abundant. All isolates produced micro and macro conidia. Microconidia were oval-ellipsoid and macroconidia three to five septate. However, in present investigation, morphological and cultural variation could not help to group the isolates properly. According to Van Der Plaats-Niterink (1981), due to interspecific variation in morphology, many isolates cannot be identified unambiguously. To understand existence of variation among the isolates of F. o. f. sp lycopersici, PCR based technique, i.e. RAPD was resorted.

The R. solanacearum produce fluidal, dull white, round to irregular colonies on sucrose peptone agar medium after 48 h of incubation (at 32^{o}C). However, virulent colonies were irregularly shaped, fluidal, dull white with slight pink to reddish center. Similar colonies of R. solanacearum were described by Kelman (1954) and Vijayakumari (2004).

Identification of root knot nematode species was made on the basis deformed roots with galls on infected plants and also perineal pattern of nematode. These studies were agreement with the study conducted by Eisenback et al. (1981).

5.4 Pathogenicity test

Pathogenicity of F. o. f. sp. lycopersici was proved by inoculating plant with spore suspension of fungus were yielded symptoms of wilt within 15 days and reisolation of fungus from inoculated plant shows similar morphological characterstics of fungus. Similar observation were made by Amini (2009).

Twenty three isolates collected from different locations showed varying degree of aggressiveness in inoculated plants: eight isolates (Fol-1, Fol-4, Fol-6 Fol-9, Fol-11, F ol-13, Fol-15 and Fol-21) showed strong virulence with 75 per cent severity and wilting symptoms were noticed 14 days after inoculation. Group second isolates (Fol-2, Fol-3, Fol-5, Fol-7, Fol-19and Fol-22) were the next strong virulent isolates producing wilting symptoms 17 to 21 days after inoculation and showed 53-75 per cent severity. Third group isolates (Fol-8, Fol-10, Fol-12, Fol-14, Fol-16, Fol-17, Fol-18, Fol-20 and Fol- 23) were less aggressive and took more time to cause symptoms (24-30 days) and severity varied from 26-50 per cent. A Similar study conducted by White (1972), by grouping F. o. f. sp. lycopersici isolates. The main aim of grouping of these isolates were to understand pathogenic variation among the isolates of F. o. f. sp lycopersici. They were detected by association with random amplified polymorphic DNA (RAPD) markers. In our study the RAPD patterns found suggested that weak and virulent pathogens could belong to the same cluster with scarce genetic distance within them. Similarly, Carmer et al. (2003) characterized genetic diversity and pathogenicity of 166 isolates of F. oxysporum obtained from common bean and sugar beet plants using RAPD analysis. They concluded that RAPD markers had only limited usefulness in correlating pathogenicity among the isolates and races.

The pathogenicity test for R.solanacearum was proved by inoculating suspension of bacterial culture ($1x10^{-8}$ cfu /ml) by root inoculation technique preparabaly at third true leaf stage. The inoculated plant started expressing wilt symptoms within 15 days as similar symptoms was described in symptomatology, and reisolation of bacteria were observed as original culture of bacteria with respective colony characters. Similar studies were conducted by Kelman (1954).

Based on pathogenicity test, twelve isolates viz. Rs-1, Rs-4, Rs-7, Rs-8, Rs-9, Rs-12, Rs-16, Rs-19, Rs-21, Rs-22 Rs-23 and Rs-24 were highly virulent. And Rs-2, Rs-5, Rs-6, Rs-10, Rs-11 and Rs-20 isolates showed medium virulence. And low virulence was found in Rs-3, Rs-13, Rs-14, Rs-15, Rs-17 and Rs-18 isolates inoculated artificially on susceptible tomato plant (Pusa ruby). The main aim of grouping of these isolates were to understand pathogenic variation among the isolates, and they were detected by association with RAPD markers. In our study, there is no clear relationship was found between relative pathogecity value observed and clustering. Gunitaleka et al. (2004) also reported that, RAPD data did not revealed clear relationship between genetic differences and the virulence of the isolates, collected from Kanday, Matale and Monaragala districts (Sri Lanka).

The pathogenicity of root knot nematode were proved by inoculating the nematode suspention by following pot culture technique at 14 days old tomato seedlings, inoculated plant started similar symptoms as described in symptomatology and reisolation of nematode yielded original characteristics of inoculated nematode. This study was in agreement with Taylor (1971).

5.5 Molecular diagnosis

5.5.1 RAPD analysis of Fusarium oxysporum f. sp. lycopersici

The molecular markers are a useful tool for assessing genetic diversity and resolving species identities, among the molecular markers, random amplified polymorphic DNA (RAPD) is increasingly being employed in genetic diversity study among different species (Williams et al. 1990). In the present investigation we studied the degree of genetic variation through the use of RAPD markers among different isolates of F. o. f. sp. lycopersici and compared the genetic relationship between them.

Of the 30 primers (OPA, OPB and OPF series) used for amplification, OPA5, OPA8, OPA10, OPB5, OPB7, OPB8, OPF2 and OPF9 showed 100 per cent polymorphism. Similarity coeffient ranged from 0.35 to 0.73. The maximum genetic similarity of 73 per cent was between Gubbi (Fol-5) and Doddaballapur (Fol-4) isolates. Whereas, least genetic similarity was observed between Fol-17 and Fol-2 isolates. A total of 75.09 per cent polymorphism was found among the isolates, indicating a high molecular variability.

The dendrogram by RAPD data revealed that, twenty three isolates differentiated into five major clusters. The genetic relation between Fol-1 and Fol-2 (Saidapur and Garag), Fol-3 and Fol-4 (Nelamangala and Doddaballapur), Fol-5 and Fol-6 (Gubbi and Hosalli), Fol-7 and Fol-8 (Bannikoppa and Lakkundi) and Fol-11, Fol-12 and Fol-13 (Arabhavi, Khanapur and Gokak) isolates may be correlated to their geographical locations as they grouped into same clusters. It can be said that some of the isolates from same geographical locations were closely related. Similar findings was reported by Sharma et al. (2006) in case of pea wilt pathogen F. o. f. sp. pisi.

Some isolates belong into RAPD cluster, viz. Fol-14, Fol-23, Fol-15, Fol-19, Fol-20, Fol-21, Fol -16 and Fol-22 isolates were found in one cluster and Fol-17 and Fol-18 isolates grouped in separate cluster. There was no apparent correlation was found between RAPD groups and geographical region which suggests that they are independent from each other, this indicates genetic diversity existing among the F. o. f. sp. lycopersici isolates collected from same geographical areas. RAPD profile indicated that geographic variation may be due to mutation in the genome of F. o f. sp. lycopersici. These findings are agreemented with Mishra et al. (2010).

It can be concluded that genetic diversity among F. o. f. sp. lycopersici isolates, from different tomato growing regions in Karnataka, was determined using RAPD primers. This finding may be useful for breeding work, as in order to test for varieties resistant to tomato wilt they need to be tested against different isolates prevalent in that particular region.

5.5.2 RAPD analysis of Ralstonia solanacearum

Polymerase chain reaction (PCR) based molecular markers are useful tools for detecting genetic variation within populations of phytopathogens. The analysis of RAPD polymorphism in single cell isolates of R. solanacearum from different districts across Karnataka revealed the occurrence of a high level of polymorphism (73.93%) indicating wide and diverse genetic base.

RAPD analysis grouped all the twenty four isolates into seven major clusters. The highest similarity degree 61 per cent was observed in Rs-7 (Hosalli) and Rs-9 (Doddaballapur) isolates. The genetic relation between Rs-1, Rs-3 and Rs-2 (Dharwad Garag and Saptapur) and isolates Rs-4 and Rs-5 (UAS Bangalore and Doddaballapur) may by correlated to their geographical affiliations as they grouped into same clusters. However, majority of isolates with different geographical locations were found in same cluster, viz. Rs-18, Rs-19, Rs-22 and Rs-23 in one cluster, where as Rs-11 and Rs-12 isolates were found in a separate cluster; isolates Rs-7 and Rs-9 were found in yet another cluster. Similarly, Rs-15, Rs-17 and Rs-24 grouped in one cluster; Rs-8, Rs-14, Rs-20, Rs-13 and Rs-16 were found in a separate cluster; and isolates Rs-10 and Rs-21 isolates grouped in yet another cluster. Thus, in respect of some isolates, phylogenetic grouping based on RAPD data did not appear to be congruent with geographical locations. It may be sumrised that the population of R. solanacearum in Karnataka is genetically heterogeneous and the interrelationship among the different isolates can be reliably and precisely explained by RAPD marker. The present studies are in accordance with Gunathilake et al. (2004).

5.5.3 Detection of Meloidogyne incognita by 18S rDNA gene amplification

The cultures of M. incognita (UAS Dharwad, Saidapur, Arabhavi and UAS Bangalore) were detected by 18s small subunit ribosomal gene amplification using two sets of primers 18s Forward primer (5′-ATGTATAAGTTTAATCGTTTTAACGA -3′) and 18s Reverse primer (5′-GTATGTACCAACTATTTAGTAGGT- 3′) and primers were specially designed for this study, PCR performed with the primer combination produced only the single expected fragment of 1.3 kb for all isolates of M. incognita. Similar work was carried out by Cliff and Hirschmann (1985) and Rammah and Hirschman (1990) who reported that isolates of M. arenaria amplified 18s fragment of 1112 bp.

5.6 Serodiagnosis

5.6.1 Serological detection of Fusarium oxysporum f. sp. lycopersici

PAbs were raised in (3-month old) white albino rabbit, using total protein extracted from mycelium of F. o. f. sp. lycopersici as antigen similar method was conducted by Srivastava and Arora (1997).

The antiserum produced in this study appears comparatively more specific than that of previous workers who used as cell homogenates or cell wall preparation as immunogen (Charudattan and De Vay, 1974; Fitzell et al., 1980). Because the antiserum obtained in this study appears to specific for F. o. f. sp. lycopersici and did not cross react with other fungal pathogens, viz. Rhizoctonia solani, Sclerotium rolfsii and Alternaria alternata.

The tube precipitation results indicated that the antiserum produced against F. o. f. sp. lycopersici was working (1:1280 dilutions). Further confirmation was done by DAC and DAS- ELISA technique.

Titre of antisera was determined by two-fold serial dilutions of antiserum from 100 to 204800. The titre of antisera against antigen was determined by DAS- ELISA. It was sensitive enough to detect the antigen up to 51200 dilutions, while the DAC- ELISA technique detected the antigen only up to 25600 dilution. Similarly Soo Bong Park et al. (2005) reported that, titre of antisera against Colletotrichum gloeosporioides antigen determined by DAC-ELISA technique, was high enough to be detectable up to x25,600 dilutions.

Reactivity of F. o. f. sp. lycopersici antiserum to the purified antigen of F. o. f. sp. lycopersici was determined using DAC- ELISA. The antigen was diluted with phosphate buffer to adjust the given concentration from 0.1 to 204.8 μg.

The result showed that, the antigens at concentrations of 3.2 µg were detected using DAC -ELISA. In contrast, antigens of 0.1 to 1.6 µg were not detected and gave a negative result. Similar results were discussed by Mahmoud et al. (2010) in their studies. Sensitivity of the antiserum ranged from 5-50 µg of Phytophthora infestans antigen.

Sensitivity of antiserum was also determined, the plates were coated with conidia of F. o. f. sp. lycopersici, serial ten-fold dilutions of conidial suspension from 10^{10} to 10^{0}/well, The polyclonal antibody had the highest level of reactivity to F. o. f. sp. lycopersici conidial antigen, Sensitivity of PAb was precise enough to detect spore concentration to as low as 50 conidia/well by DAC- ELISA technique. Similar study was conducted by Jung Han Lee et al. (2004) who reported that polyclonal antibody raised against Colletotrichum gloeosporioides was precise enough to detect spore concentration as low as 500 conidia/well by DAC –ELISA technique.

The specificity of antiserum produced against F. o. f. sp. lycopersici was tested by DAC- ELISA technique, twenty three isolates F. o. f. sp. lycopersici collected from different agroclimatic zones of Karnataka reacted positively. Among other fungi evaluated to determine the possibility of non specific cross reactions viz., Rhizoctonia solani, Sclerotium rolfsii and Alternaria alternata showed negative result against antisera of F. o. f. sp. lycopersici. The result of this study suggested that a relative specific fungal antiserum can be produced by purified antigen from lyophilized mycelium. The antiserum produced in this study appears to be more specific than antigen used like whole fungal cell homogenate or cell wall preparations as immunogens. This is in agreement with Charudattan and Devay (1972), Fitzell et al. (1980) and Gerik et al. (1987). The antiserum cross-reacted only with fungi other than F. o. f. sp. lycopersici species (0.165-0.193) .wherein, very week ELISA values (negative response) were obtained. Similar studies are also reported by Harrison et al. (1990), Kynerova et al. (1998) and Skottrup et al. (2006). Lack of specificity in the antiserum raised against against F. o. f. sp. lycopersici may be attributed to the very close taxonomical relation between F. o. f. sp. lycopersici and other Fusarium species studied. This indicated that, there are also many molecular epitopes of fungal pathogen, which are common to a F. o. f. sp. lycopersici and other Fusarium species studied. Such results were supported by Gautam et al. (1999) who reported lack of specificity in antiserum of P. infestans. There by it cross react with Pythium sp. It is due to very close taxonomical relation (shares commen molecular epitopes) between P. infestans and Pythium sp.

The rhizosphere soil of tomato collected from different tomato growing areas of Karnataka (UAS Dharwad, Saidapur, Garag, Arabhavi, Khanapur and Kolar) infested with Fusarium wilt gave positive result by DAC- ELISA- technique. Plant samples infected with F. o. f. sp. lycopersici collected from different location also reacted with antisera of F. o. f. sp. lycopersici and healthy tomato plant samples did not react. Similar results were shown by Ivana et al. (2002) wherein the antisera raised against Rhizoctonia spp. showed positive reaction with all samples of strawberry roots infected with binucleate Rhizoctonia. The reaction was absent with antigens from healthy root samples.

5.6.2 Serological detection of Ralstonia solanacearum

PAbs were developed using virulent fluidal bacterial whole cells (1 x10^4 cells /ml) as antigen in the presence of Freund's incomplete adjuvant (Robinson et al. 1995). Moreover, a lower concentration of bacterial cells, i.e. 1x10^4 cells/ml was used in a immunization of rabbit. The external layer containing glycoproteins and exopolysaccharides were believed to be the probable antigenic determinant (Stackebrandt et al., 1988; Anonymous 1997) when virulent cells were injected, and found to be toxic if given at higher concentrations, therefore only 10^4 cells/ml were used to immunize and a long-time immunization protocol was adopted to potentiate the production of antibodies.

The tube precipitation results indicated that the antiserum produced against R. solanacearum was working (1:2560 dilution). Further confirmation was done by DAC and DAS- ELISA technique.

The DAS- ELISA technique was capable of detecting R. solanacearum of 10^3 cells/ml, while the DAC- ELISA technique detected only up to 10^5 cells/ml. This indicateed that the DAS- ELISA technique was more sensitive than DAC- ELISA technique.

Similar results were observed during comparing sensitivities of the ELISA techniques using R. solanacearum by Machmud and Yadi (2008).

Twenty four isolates of collected from different agro climatic zones of Karnataka showed positive results. One other bacterial pathogen was also tested to check the cross reaction of antibody produced against R. solanacearum. Antigen of Xanthomonas sp. showed negative results using DAC -ELISA technique. Similar results were reported by Robinson (1993) while testing specificities of PAb against different strains of R. solanacearum strains and other bacterial pathogens, using the same technique.

DAC -ELISA technique was used to test the naturally infested soil and plant material collected from different regions of Karnataka. Soil from tomato rhizosphere infested with R. solanacearum showed positive result and soil collected from healthy tomato rhizosphere did not react. The technique was also able to detect R. solanacearum directly from tomato plant samples infected with R. solanacearum collected from different locations. The high value of OD_{405} (0.603-0.725) on plant samples infected with R. solanacearum indicated that the population of R. solanacearum in the plant samples were high as compared to OD value of soil sample (0.468 to 0.668) collected from different location. This may happen due to two possibilities, either the R. solanacearum population in the rhizospheres were lower than those in the plant samples or sensitivities of the DAC- ELISA technique for the detection of R. solanacearum in the soil was not optimal due to the presence of some inhibitors in soil (Stobbs and Barker, 1985; Janse, 1988 and Seal et al.,1992).

5.6.3 Serological detection of root knot nematode

Antisera were produced in female albino rabbit. Four weekly intramuscular injections with antigen preparations (homogenates of J_2 and eggs, 1mg/ml) as described by Charudattan and De Vay (1972).And antigenic properties of J_2 and eggs of Meloidogyne sp. was also reported by earlier workers Hussey (1971), Misaghi and Michael (1973).

The antisera raised against root knot nematode (M. incognita) were tested against M. incognita antigen using DAC- ELISA assay. All the four isolates collected from different regions (UAS Dharwad, Saidapur, Arabhavi and UAS Bangalore) showed positive reaction. Similar results were obtained by Liziane et al. (2005) who reported detection of Meloidogyne spp. using polyclonal antibody by Indirect -ELISA technique.

DAC- ELISA technique used to test the naturally infested soil samples (UAS Dharwad, Saidapur, Arabhavi and UAS Bangalore) also showed positive reaction. A similar study was conducted by Davis (1995) he reported polyclonal antibodies have been used for quantification of Meloidogyne sp. His results showed that polyclonal antibodies were highly sensitive and sufficiently quantify less number of nematode population per gram of soil.

FUTURE LINE OF WORK

There is no full stop to gain insight into scientific knowledge. Any amount of work does not satisfy the hunger of scientists: as problems creep in, new ideas will continue to flow and this work is not an exception. The present investigation has produced new information pertaining to the three ubiquitous vascular soil borne pathogens infecting tomato crops. The investigation has and given rise to new ideas on detection of these soil borne pathogens (Fusarium oxysporum f. sp. lycopersici, Ralstonia solanacearum and Meloidogyne incognita). Hence, the following future lines of work are being suggested.

- ✓ Development of specific (molecular and immunological) probes for detection of soil borne pathogens affecting tomato.
- ✓ Identification of the latent infection by molecular and immunological techniques
- ✓ The designing of specific primers for detection of soil borne pathogens affecting tomato directly from soil sample by polymerase chain reaction.

6. SUMMARY AND CONCLUSIONS

Tomato (Solanum lycopersicum Mill.) is one of the most widely grown vegetable crops in the world. Tomato crop is attacked by several ubiquitous soil borne plant pathogens (Fusarium oxysporum. f. sp. lycopersici, Ralstonia solanacearum and Meloidogyne incognita). All these together are limiting factors in tomato production throughout Asia. In the present investigation on molecular and serological diagnosis of these pathogens affecting tomato, the following aspects were studied.

- ✓ Soil borne pathogens affecting tomato (F. o. f. sp. lycopersici, R. solanacearum and M. incognita) were collected from different tomato growing regions of Karnataka. The aim of collection from different locations was to characterize these isolates affecting tomato by molecular and immunological diagnosis.
- ✓ It was observed that F. o. f. sp. lycopersici collected isolates caused yellowing of leaves, stunting and wilting of tomato plants. The vascular tissue of a diseased plant was dark brown, while in case of wilt caused by R. solanacearum isolates, drooping of leaves followed by the complete plant wilting within a few days was observed. Recently wilted plants were green in colour and cross section of portion of stem of the plant with bacterial wilt oozed a white, milky strand of bacterial cells in clear water. Root knot infected tomato plants showed stunted growth coupled with yellowing of leaves. When infested plants were uprooted, deformed roots with prominent multiple galls of varying sizes were noticed.
- ✓ F. o. f. sp. lycopersici produced three kinds of spores, viz. Microconidia, macroconidia and chlamydospores. Microconidia were abundant, oval-ellipsoid, straight to curved; Macroconidia, sparse to abundant, three to five septate, fusoid-subulate and pointed at both ends, and had pedicellate base. Three septate spores were predominant. Chlamydospores, were abundant, formed terminally or on an intercalary basis. Mycelia of the pathogen were white cottony to pink often with purple tinge or reddish colouration of the medium.
- ✓ Twenty three isolates of F. o. f. sp lycopersici were assigned into into three groups, on the basis of colony diameter, colony characters, sporulation, and degeree of pathogenicity. Isolates Fol-1, Fol-4, Fol-6 Fol-9, Fol-11, Fol-13, Fol-15 and Fol-21 showed abundant aerial mycelium and sporulation with maximum colony diameter (75 to 90.0mm). They showed strong virulence with 75 per cent severity and wilting symptoms were noticed 14 days after inoculation. The main aim of grouping of these isolates was to get an initial understanding variation among the isolates of F. o. f. sp lycopersici. It was further detected by employing random amplified polymorphic DNA (RAPD) markers. There was no apparent correlation is found between RAPD groups with grouping of F. o. f. sp lycopersici isolates.

The R. solanacearum isolates produced Fluidal, Irregular to round, and slimy, dull white colonies with slight pink to reddish center on TZC agar medium. Pathogenicity of the twenty four isolates were proved on their tomato hosts under artificial inoculation condition and the plants started showing wilting symptoms after 15 days of inoculation. Inoculated isolates are grouped into three groups, viz. highly virulent (Rs-1, Rs-4, Rs-7, Rs-8, Rs-9, Rs-12, Rs-16, Rs-19, Rs-21, Rs-22 Rs-23 and Rs-24), medium (Rs-2, Rs-5, Rs-6, Rs-10, Rs-11 and Rs-20), and low virulent (Rs-3, Rs-13, Rs-14, Rs-15, Rs-17 and Rs-18). And here also there was no correlation was found between morphological and pathogenic characters with RAPD clusters.

- ✓ RAPD analysis of F. o. f. sp. lycopersici revealed that maximum genetic similarity (of 73 per) cent was between Gubbi (Fol-5) and Doddaballapur isolates (Fol-4), whereas least genetic similarity was observed between Chintamani (Fol-17) and Garag (Fol-2) isolates. The dendrogram for pooled data showed five major clusters. And the genetic relation between Fol-1 and Fol-2, Fol-3 and Fol-4, Fol-5 and Fol-6, Fol-7 and Fol-8 and Fol-11, Fol-12 and Fol-13 isolates may well be correlated to their geographical locations as they grouped into same clusters. And RAPD clusters including Fol-14, Fol-23, Fol-15, Fol-19, Fol-20, Fol-21, and Fol -16 and Fol-22 isolates.

And Fol-17 and Fol-18 in RAPD cluster did not correlate their geographical affiliations with RAPD markers studied. This indicates genetic diversity existing among the F. o. f. sp. lycopersici isolates collected from different geographical area.

- ✓ The similarity co-efficient of R. solanacearum isolates ranged from 0.19 to 0.61. Maximum genetic diversity of 61 per cent was between Hosalli (Rs-7) and Doddaballapur (Rs-9) isolates, whereas least similarity (0.19 per cent) was observed between Kolar (Rs-22) and Garag (Rs-2) isolates. The data distinguished the isolates of R. solanacearum into seven major clusters. The genetic relation between Rs-1, Rs-3 and Rs-2 (Dharwad Garag and Saptapur) and Rs-4 and Rs-5 (UAS Bangalore and Doddaballapur) may be correlated to their geographical affiliations as they grouped into same clusters. And majority of remaining isolates did not fall into the same clusters, reflecting fact that the variation is independent of geographical location.
- ✓ 18s Forward primer (5′-ATGTATAAGTTTAATCGTTTTAACGA -3′) and 18s Reverse primer (5′-GTATGTACCAACTATTTAGTAGGT- 3′) was specially designed for detection of 18s small subunit ribosomal gene of M. incognita. PCR performed with the primer combination produced only the single expected fragment of 1.3 kb for all isolates of M. incognita.
- ✓ The specificity of antiserum produced against F. o. f. sp. lycopersici was tested by ELISA techniques. DAS- ELISA was more sensitive it was precise enough to detect the antigen up to 51200 dilutions, as compared to DAC- ELISA up to a dilution of 25600.
- ✓ Purified antigen of F. o. f. sp. lycopersici concentrations of 3.2 µg (or more) and (as low as) 50 conidia/well can be detected by DAC –ELISA technique.
- ✓ The DAS-ELISA technique was capable of detecting R. solanacearum 10^3 cells/ml, while the DAC- ELISA technique detected only up to 10^5 cells/ml. This indicated that the DAS-ELISA technique was more sensitive than DAC- ELISA technique.
- ✓ Isolates of R. solanacearum collected from different agro climatic zones of Karnataka showed positive reaction using DAC- ELISA techinique. This technique was also able to detect R. solanacearum directly from soil and plant sample infected with R. solanacearum.
- ✓ Four isolates M. incognita collected from different regions showed positive reaction by DAC- ELISA assay. This technique also showed positive reaction to soil sample infested with root knot nematode.

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MOLECULAR CHARACTERIZATION AND SERODIAGNOSIS OF VASCULAR PATHOGENS AFFECTING TOMATO

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ABSTRACT

Soil borne pathogens affecting tomato (*Fusarium oxysporum* f. sp. *lycopersici*, *Ralstonia solanacearum* and *Meloidogyne incognita*) were collected from different tomato growing regions of Karnataka. *F. o*. f. sp. *lycopersici* isolates, *viz.* Fol-1, Fol-4, Fol-6 Fol-9, Fol-11, Fol-13, Fol-15 and Fol-21 showed abundant aerial mycelium and sporulation with maximum colony diameter (75 to 90.0 mm): These were highly virulent. Among twenty four isolates of *R. solanacearum,* Rs-1, Rs-4, Rs-7, Rs-8, Rs-9, Rs-12, Rs-16, Rs-19, Rs-21, Rs-22, Rs-23 and Rs-24 isolates were highly virulent. RAPD analysis of *F. o*. f. sp. *lycopersici* revealed five major clusters. Maximum genetic similarity (73 %) was between Gubbi (Fol-5) and Doddaballapur isolates (Fol-4), whereas least genetic similarity was observed between Chintamani (Fol-17) and Garag (Fol-2) isolates. The similarity co-efficient of *R. solanacearum* isolates ranged from 0.19 to 0.61. Maximum genetic diversity of 61 per cent was between Hosalli (Rs-7) and Doddaballapur (Rs-9) isolates whereas least similarity (0.19 per cent) was observed between Kolar (Rs-22) and Garag (Rs-2) isolates. PCR performed with the primer combination of Forward primer (5′-ATGTATAAGTTTAATCGTTTTAACGA-3′) and 18s reverse primer (5′-GTATGTACCAACTATTTAGTAGGT- 3′) produced only the single expected fragment of 1.3 kb for all isolates of *M. incognita.* DAS- ELISA was more sensitive: it was precise enough to detect the *F. o*. f. sp. *lycopersici* antigen up to 51200 dilutions. Purified antigen of *F. o*. f. sp. *lycopersici* concentrations of 3.2 µg and 50 conidia/well could be detected by DAC –ELISA technique: The technique was capable of detecting *R. solanacearum* at 10^3 cells/ml; collected isolates of *R. solanacearum* showed positive reaction using DAC- ELISA techinique. This technique was also able to detect *R. solanacearum* directly from soil and plant sample infected with the bacterium. Four isolates *M. incognita* collected from different regions showed positive reaction by DAC- ELISA assay.