Characterization of *Ralstonia solanacearum* strains isolated from susceptible host plants

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Characterization of *Ralstonia solanacearum* strains isolated from susceptible host plants" is based on the results of investigations carried out by me in the Microbiology Programme at the, School of Biological Sciences and Biotechnology Goa University under the supervision of Dr Trupti Asolkar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Characterization of *Ralstonia solanacearum* strains isolated from susceptible host plants"** is a bonafide work carried out by Ms Nikita Nila Gaonkar under my supervision in partial fulfilment of the requirements for the award of the degree of **M.Sc. in Microbiology** in the Microbiology Programme at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

Ralstonia solanacearum is a globally widespread phytopathogenic bacterium, causing deadly wilt disease in economically important crops and ornamental plants. Classified as a 'species complex,' it encompasses heterogeneous strains, with four phylotypes known as causative agents of bacterial wilt. The term 'species complex' was first applied in 1994 to reflect its phenotypic and genotypic variation. This complex includes closely related organisms like the blood disease bacterium (BDB) and *Pseudomonas syzygii*. Studies have shown DNA-DNA homology below the 70% threshold level expected within a species, solidifying its designation as a species complex. Originating in Indonesia in 1864, it initially affected tobacco and was known as *Pseudomonas solanacearum* before being renamed *Ralstonia solanacearum* in 1995. Its impact extends globally, with various names based on geography, symptoms, and hosts affected. It infects over 400 plant species, posing a significant threat to agriculture, notably affecting crops like tomato, tobacco, potato, and banana. Symptoms manifest during the vegetative growth stage, affecting both above and below-ground plant parts. Upon infection, it infiltrates the plant's vascular system, multiplying in xylem vessels and disrupting water transport, leading to wilting and plant death. With four distinct phylotypes, its diversity and pathogenicity remain a focal point of study.

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ABBREVIATIONS USED

Entity	Abbreviations
Cell Wall Degrading Enzyme	CWDE
Room Temperature	RT
Type 3 effectors	T3E
2, 3, 5 triphenyl tetrazolium chloride	TZC
Milli litre	mL
Micro litre	µL
Polymerase Chain Reaction	PCR
Extracellular Polysaccharide	EPS
Restriction fragment length polymorphism	RFLP
Internal Transcribed Spacer	ITS
Quorum sensing	QS
Carboxy Methyl Cellulose	CMC
Type 3 Secretion System	T3SS
Type 2 Secretion System	T2SS
Hours	hrs

ABSTRACT

Ralstonia solanacearum is a globally widespread phytopathogenic bacterium, causing deadly wilt disease in economically important crops and ornamental plants.it can infect more than 400 plant species. In this study, a total of 22 isolates of *R. solanacearum* infecting solanaceous vegetables and other crops has been used. The pathogen was confirmed by PCR and the pathogenicity was tested on eggplant in greenhouse facility. The study involves characterization of various virulence factors - EPS production, twitching motility, Cell wall degrading enzymes namely pectinase and cellulase. All the isolates were found to be positive for virulence factors. Multiplex PCR and biochemical test revealed that all the isolates belong to Biovar 3 and Phylotype I. In the pathogenicity testing some isolates were found to be highly virulent and some were medium virulent.

Keywords: Twitching motility, Biovar determination, Phylotype Classification, EPS, PCR, Cell Wall Degrading Enzymes.

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Ralstonia solanaecearum is a phytopathogenic bacterium which is widespread all over the world and causes wilt disease with deadly effects on many economically important crops and ornamental plants. It is a Gram negative, soilborne pathogen and is considered a 'species complex' formed by heterogeneous strains classified into four phylotypes all of them causative agents of bacterial wilt. A species complex is defined as a cluster of closely related isolates whose individual members may represent more than one species. Gilling and Fahy in 1994 first applied this term "species complex" to *R. solanacearum* to reflect its phenotypic and genotypic variation within the species. It includes two closely related organisms, the blood disease bacterium (BDB) and *Pseudomonas syzygii* as both this were found to fall within the diversity of *R. solanacearum* (Tanghavi 1996) DNA-DNA homology experimental studies have revealed that the relatedness between isolates of *R. solanacearum* is often less than the 70% threshold level commonly expected within a species therefore *R. solanacearum* is defined as species complex (Palleroni, 1971) (Fegan and Prior, 2005). This soil and waterborne phytopathogen penetrates the plant root system, multiplies through xylem vessel, damages the host, and returns to the environment.

The first pathogenic disease caused by *Ralstonia solanacearum* was reported in Indonesia in 1864, where it caused damage to tobacco. This pathogen was first known as *Pseudomonas solanacearum* named by an American plant pathologist, smith in 1869 (Lu, 1998) then it was transferred to the genus *Burkholderia* in 1992 (Yabuuchi 1992). Again, after almost 3 years based on the results of phenotypic characterization, cellular lipid and fatty acid analysis, phylogenetic analysis it was again moved and named as *Ralstonia solanacearum* (Yabuuchi 1995)

Bacterial wilt is distributed world-wide including South America, Europe, and Asia. Based on the geographical distribution, symptoms and host affected theses disease has various names such as brown rot of potato; southern bacterial wilt; Granville wilt of potato; Moko disease in banana; slime disease; vesicular wilt or bacterial wilt etc (CABI, 2003). Along with world-wide distribution they are able to infect over 400 plant species worldwide, being a major threat to agriculture, *Lycopersicon esculentum* (tomato), *Nicotiana tabacum* (tobacco). *Solarium melongena (*aubergine*). Solarium tuberosum (*potato*), Musa paradisiaca (*banana*), Heliconia* are the plants, mainly affected by the pathogenic disease.

R. solanacearum affects the plants during their vegetative growth stage and affects plant parts like vegetative organs, stem, fruits, seeds and eventually the whole plant. Environmental condition also plays crucial role in wilting of the plant. Symptoms can be seen in both above and ground organs of Solanaceae plants. There are external and internal symptoms which can be seen on the host, external symptoms include wilting, stunting, yellowing of the foliage, drooping of leaves showing leaf epinasty. The most frequent internal symptoms include discolouration of vesicular tissue (mainly xylem) at early stages of infection and as the disease develops portion of pith and cortex also shows discolouration until complete necrosis (Kelman 1953; Smith 1920)

Using soil as a medium *R. solanacearum* can infect the host through plant root and cause disease. After penetration, it reaches the vascular system of the plant then it propagates and multiplies in large numbers in the xylem vessels, and a large amount of extracellular polysaccharide is secreted, which disturbs the water transport system inside the plant and eventually leading to wilt and death of the plant (Kwak et al, 2018).

R. solanacearum species complex has four different phylotypes based on its geographical distribution. Phylotype I is of Asian origin. Phylotype II is of central and south American origin,

phylotype III of African origin and phylotype IV is of Indonesian origin however phylotype I and II is dispersed worldwide but phylotype III and IV remains in their centres of origin (EPPO 2024). Since *R. solanacearum* is so diverse and highly pathogenic this study focuses on the diversity and pathogenicity of this pathogen.

1.2 AIM AND OBJECTIVES

Aim: Characterization of *Ralstonia solanacearum* strains isolated from susceptible host plants.

Objectives:

1. Screening of *R. solanacearum* isolates for various virulence factors.
2. Genomic confirmation and classification of *R. solanacearum* isolates.
3. Determination of Pathogenicity of *R. solanacearum* isolates through plant assays.

1.3 HYPOTHESIS

Since the organism is identified as *R. solanacearum* species complex, this study aims at understanding the diversity in pathogenicity of *R. solanacearum* strains isolated from various geographical locations.

This study will create a base for characterizing various virulence factor possessed by *R. solanacearum* strains.

1.4 SCOPE

Understanding the various virulence factors which are present in *R. solanacearum.* Checking for the presence of this virulence factors will help in understanding their mechanism of action and the role played by each of them in causing the disease.

Investigating *R. solanacearum* strains, isolated from multiple hosts from various geographical locations will help in understanding the intensity of pathogenicity expressed by them. The

study will also help in classifying the organism using phylotype and biovar based classification. This work can be a base line for developing wilt management strategies.

Chapter 2: Literature review

Phytopathogens present in soil cause severe losses to plants every year. Among them, *Ralstonia solanacearum*, because of its destructive nature, is the world's second most damaging bacterial phytopathogen (Wang et al, 2023). Because of the variability in *R. solanacearum* strains, it is very difficult to control bacterial wilt (Bhunchoth et al, 2015). Xylem, a complex water transporting tissue, made up of a variety of cell types, both dead (tracheid) and living (parenchyma). Appearance of wilting in plants is caused due to blockage in xylem which in-turn caused due to exopolysaccharide a substance produced by Ralstonia (Ingel et al, 2021).

2.1 DIVERSITY OF *R. solanacearum*

Chauhan et al, (2021) conducted a field survey on the status of bacterial wilt caused by *Ralstonia solanacearum* of solanaceous crops in Himachal Pradesh. They observed significant variation in bacterial wilt incidence in different areas. They reported highest wilt incidence (90 – 100%) in Arka variety of brinjal at Palampur district and in different varieties of tomato at Nayasar, Palampur and Gaura districts while capsicum California wonder variety was also reported to found 60 – 90% wilt incidence.

Bholanath et al, (2014) have conducted a field survey during 2004 to 2007 throughout the region of west Bengal, and incidence of some economically important crops (tomato, potato, eggplant, chillies, banana, ginger etc) and twelve wild plants were recorded with medium to high bacterial wilt intensity. They confirmed the bacterial wilt disease by ooze test and other biochemical test in laboratory. During experimental period they found a sharp relationship between disease intensity and meteorological factors, they noted that crops transplanted during summer months were more susceptible to the infection by *R. solanacearum* than those transplanted during cooler months. In most cases they observed wilting process was started

from the month of march and maximum wilt intensity was seen during august to September and necrosis of such plants were ceased at the end of October.

A wide variation exists between the strains of *R. solanacearum* they have been divided into five races and five biovars depending on the host range and their biochemical properties (Hayward 1964). Race-1 found in tropical areas of the world and has a high temperature optimum (35°C) it affects solanaceous crops, weeds, and many other hosts in plant species. Race 2 occurs mostly in tropical areas of south America and causes wilt of banana and *heliconia.* Race III is not highly virulent to solanaceous crops though it affects tomato, potato, eggplant and on capsicum, it is often asymptomatic however Race IV infects ginger and Race V infects *Morus* plants (Pradhanang 2000)

2.2 BIOVAR DETERMINATION

Metabolic activity of *R. solanacearum* is characterized based on its utilization and/or oxidization of 3 hexose alcohol (mannitol, sorbitol and dulcitol) and three disaccharides (lactose, maltose and cellobiose) this is defined as biovar characterization and it is classified in five biovars (Hayward, 1964) Biovar I cannot utilise any of the hexose alcohols and disaccharides. Biovar II includes strains of *Ralstonia* that utilizes hexose sugar but does not utilise any of the disaccharides. Biovar III utilizes both (hexose alcohols and disaccharides). Biovar IV belonging strains utilizes only disaccharides and lastly biovar V utilizes only one hexose alcohol that is mannitol and all the disaccharides.

2.3 TYPE 3 SECRETION SYSTEM

Type 3 secretion system (T3SS) is essential for pathogenicity of *R. solanacearum* and many other pathogens, which injects effector proteins into plant cell and is encoded by hrp gene clusters (Genin & Boucher 2004). It is required at different stages of infection including efficient root cortex colonization, multiplication within the xylem etc. Type II and type III

Secretion system both are central to pathogenicity which serve to export large repertoires of pathogenicity effectors (Poueymiro & Genin 2009). Type 3 effectors (T3E) proteins also influence host cell functions. Plants have evolved specific resistance called guard proteins in response to pathogen attack that able to recognize the action of some T3Es historically called as “avirulence factors” which leads to the T3E-triggered immunity which mainly results in the HR (hypersensitive response) (Jones & Dangl 2006). In *R. solanacearum* three of such avirulence factors has been characterized till date which are the avrA gene, PopP1 or PopP2 protein. In fact, inoculation of hrp mutants directly in the stem vascular system does not lead to disease symptom development hence in such cases T3SS plays an important role in pathogenesis. Intriguingly, the *R. solanacearum* T3SS also appears to reprogram organogenesis of the root system by inducing the formation of root lateral structures that are sites of bacterial multiplication (Zolobowska & Van 2006).

To obtain nutrition and energy most of the Phytopathogenic bacteria have often developed enzymes to hydrolyze plant cell wall components, which are further involved in early stages of the infective process, favoring the entry and advance of the pathogenic agent in host tissues (Boucher et al, 2001) likewise, *R. solanacearum* produces several plant cell wall-degrading enzymes, secreted via the type two secretion system (T2SS) (Huang & Allen 1997; & Tans-Kersten et al, 1998) such as β-1,4-cellobiohydrolase (CbhA) and some pectinase as one β-1,4-endoglucanase (Egl) (Schell 1997; Roberts 1988), one endopolygalacturonase (PehA), two exopolygalacturonases (PehB and PehC) (Huang Q & Allen 1997; Schell 1997), and one pectin methyl esterase (Pme). A 43-kDa protein Egl of *R. solanacearum* has proved to be involved in pathogenicity (Roberts DP 1988). Inactivation of these genes mainly Egl, PehA and PehB revealed that each contribute to *R. solanacearum* virulence, and a deficient mutant lacking the six enzymes wilted host plants more slowly than the wild-type (Liu et al, 1988). Pectin catabolism does not significantly contribute to bacterial fitness inside the plant (González &

Allen 2003) it seems that cellulase and pectinolytic activities plays a very important role in host colonization than for bacterial nutrition (Valls et al, 2006). Hence, *R. solanacearum* hydrolytic enzymes are thought to be involved in pathogenicity in of their host (Hikichi et al, 2007).

2.4 EPS (Exopolysaccharide)

Exopolysaccharides (EPSs) is substance produce by Several phytopathogenic bacterial species either in pure culture or during in planta multiplication. Although it is related to pathogenicity, but it not fully understood whether EPSs take active part in symptom production or if they indirectly favor infection (Schell, 2000). It has been reported that in *R. solanacearum* all wild-type Virulent strains (mucoid colonies) produce EPS (Kelman 1954) while EPS-deficient mutants (non-mucoid colonies) are avirulent. Since EPS composition varies among different strains of *R. solanacearum* it is considered as highly heterogenous (Drigues 1985). The main virulence factor is an acidic, high molecular mass extracellular polysaccharide (EPS I), a long (>106Da) polymer with a trimeric repeat unit of N-acetyl galactosamine, 2-N-acetyl-2-deoxy-L-galacturonic acid, and 2-N-acetyl-4-N-(3-hydroxybutanoyl)-2-4-6-trideoxy-D-glucose (Orgambide et al, 1991). EPS I is more than 90% of the total *R. solanacearum* EPS produced, and approximately 85% appears as a released, cell-free slime, whereas 15% has a cell surface-bound capsular form (Schell 2000). In studies carried out with EPS I-deficient mutants, it was found that EPS I caused wilting in infected plants (McGarvey et al, 1998). In planta, EPS would probably act by occluding xylem vessels, interfering directly with normal fluid movement of the plant, or by breaking the vessels due to hydrostatic overpressure. On the other hand, EPS I might also favor stem colonization by the pathogen, since EPS I-deficient mutants were shown to multiply more slowly, and colonized poorly the stem of infected plants (Saile et al, 1997). Thus, EPS I would be contributing to minimizing or avoiding the recognition of bacterial surface structures such as pili and/or lipopolysaccharide by plant defense mechanisms (Araud-

Razou 1998). In *R. solanacearum*, EPS is thought to be the main factor accounting for the virulence of the pathogen although it might take part mainly in late stages of the process, modulating disease severity rather than the infective ability of the bacterium as EPS-deficient mutants can infect and multiply to some extent in planta without inducing wilting symptoms.

2.5 MOTILITY

Motility is transiently expressed during *R. solanacearum* growth and can produce one to several polar flagella (Clough et al, 1997), which provide it with varies types of motilities such as swimming motility and twitching motility. However, to observe this motility, cell density should be $>10^6$ and $<10^9$cfu/ml This would be related to the culture age, so that high numbers of motile cells could be obtained in exponential phase, whereas in stationary phase there would be a majority of non-motile (Clough et al, 1997). Thus, it was reported that motility was needed to effectively invade and colonize the host, although cells were nonmotile in host xylem vessels (Tans et al, 2001). It was also found that cells from wilted plants were non-motile but, became motile after a few hours in fresh medium (Mao & He 1998). *R. solanacearum* exhibits twitching motility over solid surfaces and this form of motility consists of a flagella-independent translocation requiring retractile type IV pili (fimbriae), which is present on *R. solanacearum* which makes it being related to twitching motility, adherence to surfaces and natural transformation (Kang et al, 2002). *R. solanacearum* variants having mutations in type IV pili were markedly less virulent on host plants, and consequently motility, adherence and/or type IV pili appear to have a significant role in *R. solanacearum* pathogenesis. (Liu et al, 2001)

2.5 GENOME OF *R. solanacearum*

The genome of *R. solanacearum* has a size of 5.8 Mb with a high G+C content (average value of 67%) and a coding potential for approximately 5,120 proteins. It is divided into two independently replicating circular replicons: a 3.7 Mb chromosome and a 2.1 Mb mega plasmid

(Genin & Boucher 2004). The genomes harbour many transposable elements that may take active part in acquisition, loss, and alteration of genetic material, and so contributing to generate genomic variation, there are also various genes harboured by chromosomes that encodes for basic cellular functions, while the mega plasmid encodes many essential genes for pathogenicity and genes that may be related to the overall fitness of the bacterium.

2.6 QUORUM SENSING

Quorum sensing also plays a role in virulence of *Ralstonia solanacearum*. Quorum sensing is a cell density dependent bacterial communication system, when *Ralstonia* enters xylem cells, it multiplies and reaches high cell densities, which activates a set of virulence factors through quorum sensing. In *R. solanacearum* 3-hydroxy palmitic acid methyl ester (3OH-PAME) is the main quorum sensing molecule governing the expression of virulence factors (Achari & Ramesh 2015). QS inducers are organic small molecules in Gram-negative bacteria. At low cell densities, the level of QS signal is not sufficient to activate the QS system. However, at the bacterial population increases, the level of signal increases. Once it reaches a threshold, the QS system is activated, resulting in transcriptional reprogramming that alters bacteria behaviour including biofilm formation, toxin production, EPS, motility and production of other virulence factors (Rivera et al, 2023). Quorum quenching can be used as an alternative for control of plant diseases by cloning genes encoding quorum quenching enzymes in bacterial biocontrol agents. Quorum quenching enzymes can play an important role in maintaining plant health along with other soil enzymes and antimicrobial peptides secreted by rhizosphere bacteria (Achari & Ramesh 2018)

2.7 BIOVAR, RACE AND PHYLOTYPE CLASSIFICATION OF *R. solanacearum*

Biovar typing and race assessment are methods used for assessing the diversity of *Ralstonia* strains. However, genetic evidence had showed that phenotypically-based schemes are not

sufficient to encompass the diversity of strains represented in the species *Ralstonia* hence (Prior and Fegan 2004) presented a classification system based upon phylogenetic analysis of sequence such as 16S-23S internal transcribed spacer (ITS) region, the endoglucanase gene and the mutS gene based on the data obtained they sub divided *Ralstonia solanacearum* species complex into 4 monophyletic cluster of strains, termed phylotypes. Strains within each phylotype is broadly originate from the same geographical area.

All strains belonging to Biovar 3, 4, and 5 and strains are isolated primarily from Asia are included in phylotype I. Strains belonging to Biovar 1, 2 and 2T isolated primarily from America and *R. solanacearum* race III potato pathogens, which has a worldwide distribution, and the race 2 banana pathogen all are members of phylotype II. Strains primarily isolated from Africa and surrounding islands were included in phylotype III and belong to Biovar 1 and 2T. Strains isolated primarily from Indonesia belonging to Biovar 1, 2 and 2T were classified into phylotype IV. Each phylotype is composed of several groups of strains with a highly conserved sequence termed sequevars.

Poussier et al, (1999) assessed genetic diversity of *Ralstonia solanacearum* among the world. They used 120 strains of *Ralstonia solanacearum* and amplified fragments of the hrp gene region by using restriction fragment length polymorphism (RFLP) method. Five amplified fragments are found to be specific to *Ralstonia solanacearum*. They reported 15 different profiles among the 120 strains and distributed them in 8 clusters using hierarchical cluster analysis. Strains were included in each cluster that belongs to single biovar except for strains of biovar 3 and biovar 4, which could not be separated. However, biovar2 and strains of biovar3 and 4 are gathered in cluster 1 and 2 respectively while biovar 1 strains showed a rather large diversity as they were distributed in 5 clusters. They also confirmed the results of previous studies which split of species into an "Americanum" division grouping biovars-1 and 2 strains and an "Asiaticum" division including biovars-3 and 4 strains through PCR-RFLP analysis of

hrp gene region. However, the present study showed that most of the biovar 1 strains be originating from African countries which were included in separate cluster belongs to “Asiaticum” rather than "Americanum" division.

The reports on physiological variations of the bacterium revealed that there were variations in the physiological races/biovars of solanacearum strains on eucalyptus trees. And the Eucalypt-infecting strains in Australia (Akiew and Trevorrow 1994), China (Lin et al, 1993) and South Africa (Coutinho et al, 2000) were reported to belonged to race-I/biovar-3, whereas those in South America were reported to belong to race-I/ biovar-1 (Dianese et al, 1990). In south China, Lin et al, (1996) collected 55 isolates from the epidemic regions of eucalypt bacterial wilt, and most of them were identified as race-I, biovar-3, that confirmed the previous results of Wu and Liang (1988).

R. solanacearum due to its variability affects a wide range of host mainly solanaceous vegetable crops:

2.8 SOLANACEOUS VEGETABLE CROPS

Solanaceous vegetable plants include tomato, eggplant, chillies, and potato which plays a very important role in community and commercial agriculture in all the parts of India and worldwide. These crops are cultivated in large amount as they are rich source of essential vitamins and minerals for daily consumption by humans. These vegetable plants are threatened by number of disease-causing biological agents, bacteria is a cause of most of the diseases to these plants.

2.8.1 Tomato

Lycopersicon genus includes 9 tomato species which is now included in genus solanum based on molecular taxonomy. The cultivated tomato (*Lycopersicon esculentum*) was introduced into Europe after Spanish conquest of Mexico in 1521, it was then spread to all other continents and

now is the second most important solanaceous crop after potato. Tomatoes are used for soups, ketchups, salad, sauces, pickles and in many other ways.

2.8.2 Potato

Potato is the world's third food crop after wheat and rice in terms of production. There are approximately 180 tuber-bearing *solanum* species which are indigenous to Latin America. It is the most useful and important crop in the *Solanaceae* family. They occur in wide range of habitats from Mexico to Chile and worldwide. It is considered as the "king" in food staples. India is their fifth largest producer of potato in the world.

2.8.3 Eggplant

Eggplant is the only crop belonging to Solanaceae family that is indigenous to southeast Asia. It is popular vegetable crop grown in subtropical and tropical areas. It is also known as Brinjal or Guinea squash. It can be grown in prevailing climatic conditions of south India and the deccan plateau.

2.8.4 Chillies

Capsicum is native to tropical and temperate Americas. This genus has great economic importance as it includes vegetables and spices which is cultivated and consumed worldwide. *Capsicum annuum* complex (*C. annuum*, *C. chinense* and *C. frutescens*) is economically most important species. (Carrizo et al, 2016). Chilli is major crop of India and is used in almost all country. The major chilli growing states of India included Andhra Pradesh, Karnataka, Maharashtra, Tamil Nadu and Orissa.

As this phytopathogen has a wide range of diversity and affects more than 200 species it is very important to control bacterial wilt, hence this this study focuses on diversity of this pathogen and pathogenicity.

Chapter 3: Methodology

3.1 SAMPLE COLLECTION

Field diagnosis of disease plants was done by critically observing the bacterial wilt samples. Plants with wilted symptoms were identified and plant materials like stem, roots were collected in clean plastic bag. Twenty-one bacterial samples isolated from various pathogenic plant samples were received from Plant Pathology Laboratory, ICAR-CCARI, Goa.

3.2 BACTERIAL OOZE TEST

Bacterial ooze test is a confirmatory test at laboratory conditions. The plant materials collected from the crop fields were washed thoroughly with running tap water to remove the soil debris, then cut into about 1cm pieces using clean sharp blade or knife. The sample were then tested for the bacterial stream by dipping the cut ends in test tube containing 5-10 ml distilled water (Thakur et al, 2020)

3.3 ISOLATION OF *R. solanacearum*

The sample (1 cm) used for bacterial ooze test was used for isolation. In a sterile petri plate under sterile condition 2 drops of sterile distilled water was taken and the sample was kept inverted and pressed onto the drop of water to accumulate the bacterial stream in the water. Using sterile nichrome loop, 1 loopful from these drops were taken and streaked on BG medium supplemented with 1% TZC (2, 3, 5 triphenyl tetrazolium chloride). 100 micro litre TZC solution after syringe filtration was added to autoclaved 100ml BG media. The plates were incubated at 28ºC ± 2°C for 24-48 hrs. The virulent colonies in the medium characterized by dull white colour, fluidal, irregularly round with light pink centres were further streaked on TZC medium to get pure colonies of the bacterium (Ramesh et al, 2014)

3.4 PRESERVATION OF CULTURE

1. Water stock

   Under sterile condition, in sterile cryo vial 5-6 loopful of culture was mixed in 1 ml distilled water until it completely dissolves in water. The tubes were maintained at room temperature until further use.

2. Glycerol stock

   Under sterile condition, in sterile cryo-vial add 600µl 60% glycerol and 400µl BG broth was added. To this the culture from the plate was added by scrapping maximum amount of culture possible and resuspended completely. The vials were maintained at -20-degree celcius until further use.

3.5 BIOVAR DETERMINATION

10% solutions of carbohydrates (mannitol, maltose, sorbitol, lactose, dulcitol, cellobiose) in 10ml amounts were prepared (Heating may be required for dissolving the sugars). The hexose alcohols (Dulcitol, Sorbitol, mannitol) are stable and can be autoclaved. The disaccharides (Maltose, Cellobiose, Lactose) are heat labile and should be filter sterilised.

Basal media broth (without agar) was prepared. The final media was done by mixing 0.9ml of basal media and 0.1ml of carbohydrate solution. Two replicates of each were maintained, to this final media 100µl of culture was added. Tubes were maintained at RT and checked for colour change from blue to yellow after 48hrs.

Table 3.1: Biovar determination, adapted from Thakur et al, 2020

	Biovar 1	Biovar 2	Biovar 3	Biovar 4	Biovar 5
Maltose	-	+	+	-	+
Lactose	-	+	+	-	+
Cellobiose	-	+	+	-	+
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-
Dulcitol	-	-	+	+	-

3.6 VIRULENCE FACTORS

3.6.1 Screening of enzymes

I. Pectinase Assay: *R. solanacearum* culture was spot inoculated on pectin agar plates and incubated at RT for 48 h. To visualize pectinase activity the plates were flooded with 1% CTAB solution and incubated for 30 mins. Pectin degradation was observed as a clear halo around the colony.

II. Cellulase Assay: *R. solanacearum* culture was spot inoculated on carboxy methyl cellulose agar plates and incubated for 48h at RT. The plate was stained with 0.1% Congo red solution for 15 min followed by destaining with 1 M NaCl solution. Degradation of cellulose was observed as yellowish halo on a red background.

3.6.2 Twitching motility

Twitching motility was checked by spot inoculating *R. solanacearum* culture (10^{-8}, 10^{-7} and 10^{-6} dilution) on BG media plates. Dilutions were made by adding a loopful of culture in 1ml of distilled water and diluting it up to 10^{-8}. The plates were checked for twitching motility after 9 hrs of incubation under microscope (Corral et al, 2020)

3.6.3 EPS production

EPS production was observed by streaking *R. solanacearum* culture on BG media plates and incubating the plates at room temperature for 48hrs.

3.7 DNA EXTRACTION

DNA isolation was carried out using overnight grown cells of *R. solanacearum* maintained at 150 rpm at room temperature (RT). The culture (1.5 mL) was centrifuged at 10000 rpm for 10 min and the supernatant was discarded. The cell pellet was resuspended in 600 µL of lysis buffer, mixed thoroughly and incubated at 37°C for 1 h. To this 100 µL of 5 M NaCl was added, mixed thoroughly, followed by 80 µL of CTAB/NaCl solution and incubation at 65°C for 10 min. Equal amount of phenol/chloroform/isoamyl alcohol was added, mixed thoroughly, and centrifuged at 10000 rpm for 10 min. The aqueous layer was transferred to a fresh tube and equal amount of chloroform/isoamyl alcohol was added. The tubes were mixed thoroughly and centrifuged at 10000 rpm for 10 min. The aqueous layer was transferred to a fresh tube and the nucleic acids were precipitated in 0.6 volume of isopropanol. The tubes were mixed thoroughly and centrifuged at 10000 rpm for 10 min. The precipitated DNA was washed with 70% ethanol, dried and the pellet was re-dissolved in 50µL of TE buffer. The DNA was quantified using Nano drop-1000, Thermo fisher scientific, USA and maintained at - 30°C for subsequent use. The DNA was diluted to 50 ng/µL and used for subsequent PCR amplification and agarose gel electrophoresis.

3.7.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using horizontal gel electrophoresis apparatus (Tarson Products Pvt. Ltd., India) as per manufacturer‘s instructions. Agarose gel (0.8%) was casted using 0.5 μgmL^{-1} of ethidium bromide in 1 X TAE buffer. DNA sample (5 µL) was

mixed with 1 µL of 6 X DNA loading buffer (Thermo Scientific, USA) and loaded in the agarose gel submerged in 1 X TAE buffer. Electrophoresis was performed at 80 V and the DNA bands were visualized and documented with MultiImage Light Cabinet (Alpha Innotech Corporation).

3.8 PCR- POLYMERASE CHAIN REACTION CONFIRMATION OF *R. solanacearum* ISOLATES.

PCR conformation of R. solanacearum isolates were determined by using 759/760 primers (Opina et al, 1997). Reaction mixture of 20 microlitre contains 10 microlitre Go Taq (master mix), 1 µM of each primer, 6µl of PCR water, and 2 µl of template DNA. PCR conditions set were: initial denaturation at 94 °C for 3 min, 53 °C for 1 min 72 °C for 1 min 30 sec, followed by 30 cycles of denaturation at 94 °C for 15 sec, annealing at 60 °C for 15 sec and extension at 72 °C for 15 sec and the final extension at 72 °C for 5 min. PCR products were separated using agarose gel containing 6.0µL of ethidium bromide.

3.8.1 Phylotype-specific-multiplex PCR amplification

Phylotype affiliation was determined by multiplex PCR using a set of phylotype-specific primers in a 25 µL reaction mix performed in Mastercycler Pro (Eppendorf, GmBH) using the following cycle: initial denaturation of 96ºC for 5 min; 30 cycles of 94ºC for 15 sec, 59ºC for 30 sec, 72ºC for 30 sec and final extension of 10 min at 72ºC. Template DNA of *R. solanacearum* was provided (fig 3.11) PCR products were separated using 1.2% agarose gel containing 6.0µL of ethidium bromide (Gaitonde & Ramesh 2014).

3.9 PATHOGENICITY OF *R. solanacearum*

25-30 days old eggplant seedlings were used for testing pathogenicity. Experiment was carried out in greenhouse facility, whose temperature was maintained at 30°C and eggplant seedings were transplanted into pots containing pot mixture. Inoculum was prepared by growing *R.*

solanacearum in 20 ml BG broth for 48hrs. Culture was centrifuged at 10000 rpm for 10 mints and the pellet was resuspended in 20ml distilled water. The culture was further diluted using distilled water and the OD (optical density) at 600 nm was made to 0.1 (5×10^8 cells). Seedlings were inoculated by pouring 10 ml of culture at the base of each seedling by drench inoculation method. Two replications were maintained for each isolate. The inoculated plants were watered daily and observed every day for the occurrence of wilt till 15 days (Ramesh et al, 2014).

RESULT

SAMPLE COLLECTION**:** Wilted plant sample was collected from agricultural field.


Fig 3.1: Tomato showing wilting symptoms in agricultural field.

BACTERIAL OOZE TEST: Bacterial stream by dipping the cut ends in test tube containing 5-10 ml distilled water was observed.

Fig 3.2: Root of wilted sample


Fig 3.3: Milky bacterial exudation from cut end of tomato stem

ISOLATION OF *R. solanacearum:* Two isolates of *R. solanacearum* were isolated from wilted plant (eggplant) in Goa, on BG medium supplemented with TZC, after 48 hrs of incubation the colonies were selected and purified based on the fluidal pink centre colonies having whirling pattern.

Table 3.2: *R. solanacearum* isolates

Sr no.	**Culture code**	**Crop**	**Location**	**Place of Isolation**
1	Rs-13-382	Tomato	KVK Farmers Field Diwar	ICAR, Goa
2	Rs-13-383	Chilli	KVK, Diwar	ICAR, Goa
3	Rs-13-384	Chilli	KVK, Diwar	ICAR, Goa
4	Rs-13-385	Capsicum	KVK, Diwar	ICAR, Goa
5	Rs-13-386	Chilli	KVK, Diwar	ICAR, Goa
6	Rs-13-387	Ginger	KVK, Old Goa	ICAR, Goa
7	Rs-15-391	Tomato	KVK, polyhouse Sangolda.	ICAR, Goa
8	Rs-17-392	Brinjal	KVK	ICAR, Goa
9	Rs-22-396	Potato	Sangolda	ICAR, Goa
10	Rs-22-398	Potato	Salgini	ICAR, Goa
11	Rs-22-400	Potato	Salgini	ICAR, Goa
12	Rs-22-402	Potato	ICAR, KVK	ICAR, Goa
13	Rs-22-403	Potato	Belgaum	ICAR, Goa
14	Rs-22-405	Potato	Salgini	ICAR, Goa
15	Rs-22-406	Potato	ICAR, KVK	ICAR, Goa
16	Rs-23-408	Chilli	ICAR, field	ICAR, Goa
17	Rs-23-411	Tomato	Tamil Nadu	ICAR, Goa
18	Rs-23-414	Marigold	Sattari	ICAR, Goa
19	Rs-23-416	Tomato	Coimbatore	ICAR, Goa

20	Rs-23-417	Tomato	Coimbatore	ICAR, Goa
21	Rs-23-418	Tomato	Coimbatore	ICAR, Goa
22	Rs-24-419	Tomato	ICAR, field	Goa University
23	Rs-24-420	Eggplant	ICAR, Field	Goa University

BIOVAR DETERMINATION**:** Based on the utilization of disaccharides and oxidation of hexose alcohols, it is indicated that all the *R. solanacearum* isolates belongs to biovar 3.

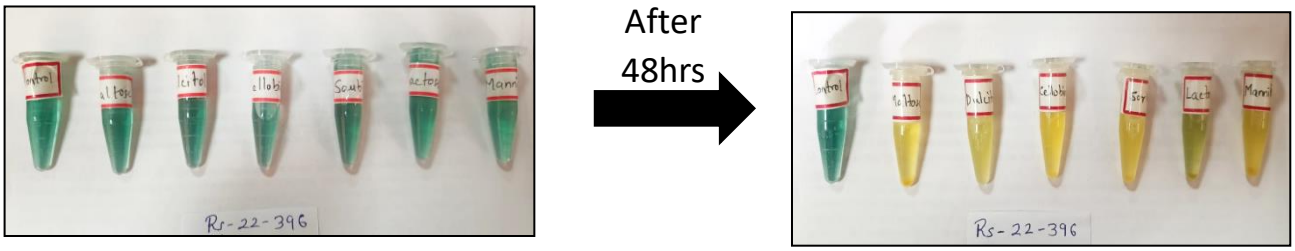

Fig 3.4: Biovar determination of Rs-22-396 isolate.

Table 3.3: Biovar determination of 22 *R. solanacearum* isolates.

Culture code	Disaccharide			Hexose alcohols		
	Maltose	Lactose	Cellobiose	Mannitol	Dulcitol	Sorbitol
Rs-13-382	+	+	+	+	+	+
Rs-13-383	+	+	+	+	+	+
Rs-13-384	+	+	+	+	+	+
Rs-13-385	+	+	+	+	+	+
Rs-13-386	+	+	+	+	+	+
Rs-13-387	+	+	+	+	+	+
Rs-15-391	+	+	+	+	+	+
Rs-17-392	+	+	+	+	+	+
Rs-22-396	+	+	+	+	+	+

Rs-22-398	+	+	+	+	+	+
Rs-22-400	+	+	+	+	+	+
Rs-22-402	+	+	+	+	+	+
Rs-22-403	+	+	+	+	+	+
Rs-22-405	+	+	+	+	+	+
Rs-22-406	+	+	+	+	+	+
Rs-23-408	+	+	+	+	+	+
Rs-23-411	+	+	+	+	+	+
Rs-23-414	+	+	+	+	+	+
Rs-23-416	+	+	+	+	+	+
Rs-23-417	+	+	+	+	+	+
Rs-23-418	+	+	+	+	+	+
Rs-24-419	+	+	+	+	+	+

EPS production**:** Presence of white fluidal colonies with whirling pattern was observed after isolating *R. solanacearum* on BG medium at 30^{o}C for 48hrs.

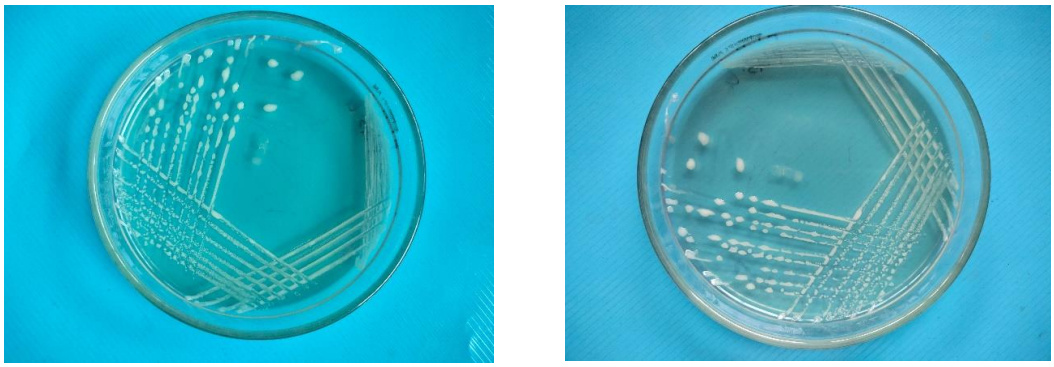

Fig 3.5: EPS production on BG media

SCREENING FOR CWDE (CELL WALL DEGRADING ENZYMES)

Pectinase activity: A clear halo around the colony was observed after flooding the plates with 1% CTAB solution and incubating it for 30 mints.

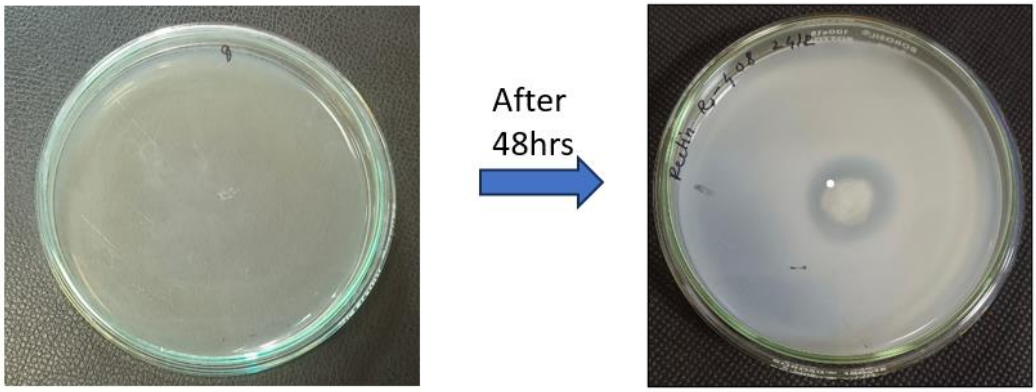

Fig 3.6: Degradation of Pectinase was observed as clear halo around the colony.

Cellulase activity: After 48hrs of incubation at 30^0C the plates were stained with 0.1% Congo red solution for 15 min followed by destaining with 1 M NaCl solution, degradation of cellulose was observed as yellowish halo on a red background.

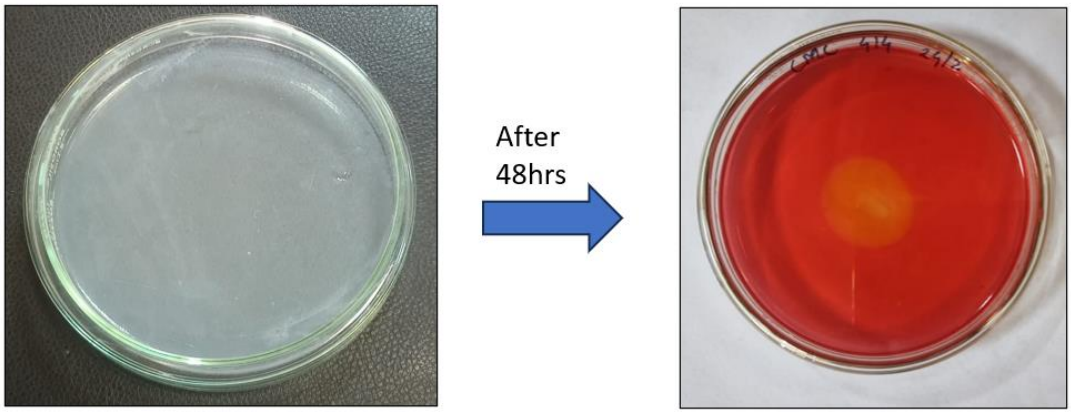

Fig 3.7: Degradation of cellulose was observed as yellowish halo on a red background.

TWITCHING MOTILITY: The plates were observed for twitching motility under Olympus CX41FX model microscope after 16hrs of incubation at RT.

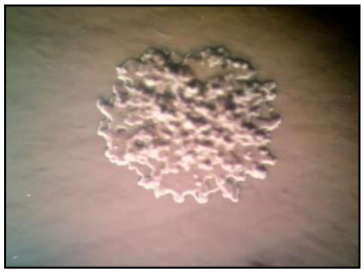

Fig 3.8: Twitching motility after 16hrs of

STAGES OF TWITCHING MOTILITY:

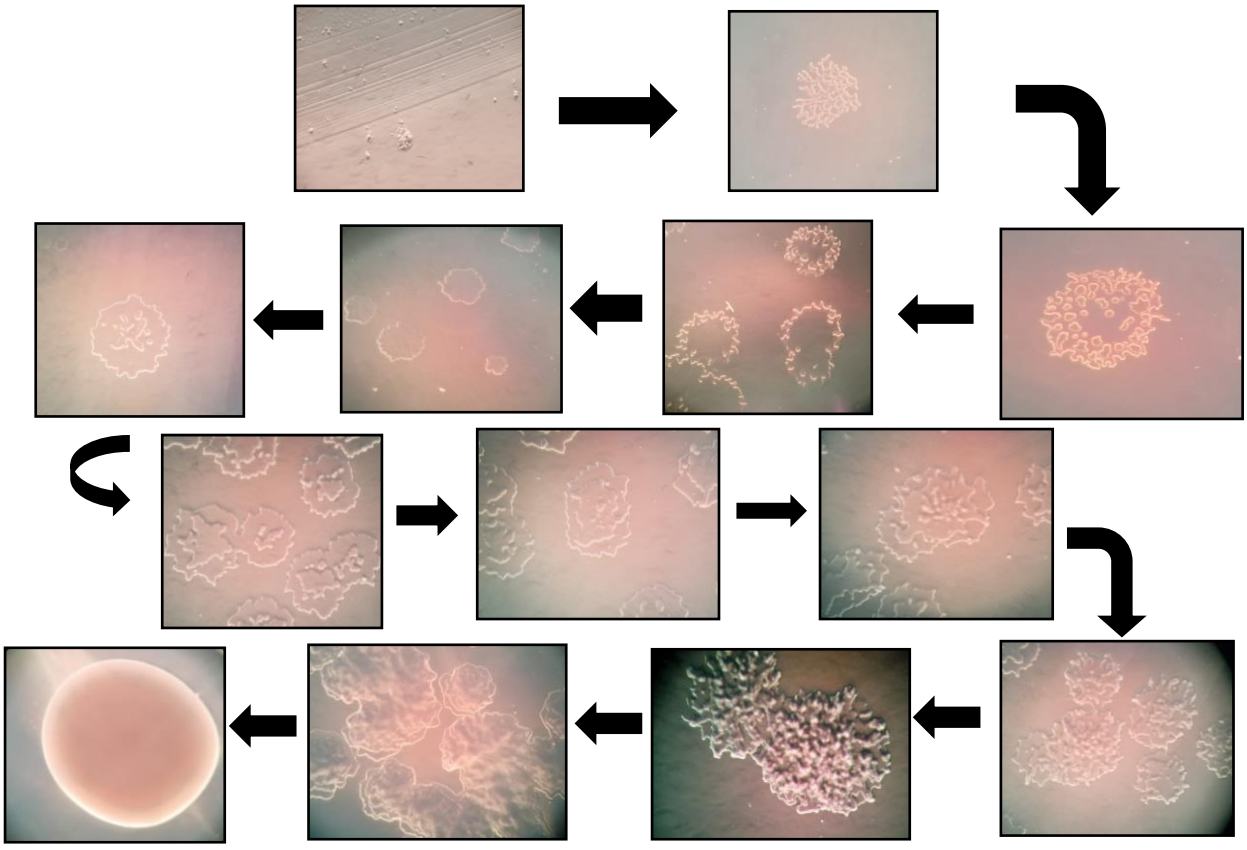

Fig 3.9: stages of twitching motility

Table 3.4: virulence factors of *R. solanacearum* isolates

Sr no.	**Culture code**	**Twitching motility**	**Enzymes (cellulose & pectin) degradation.**	**EPS Production**
1	Rs-13-382	+	+	+
2	Rs-13-383	+	+	+
3	Rs-13-384	+	+	+
4	Rs-13-385	+	+	+
5	Rs-13-386	+	+	+
6	Rs-13-387	+	+	+
7	Rs-15-391	+	+	+
8	Rs-17-392	+	+	+
9	Rs-22-396	+	+	+
10	Rs-22-398	+	+	+
11	Rs-22-400	+	+	+
12	Rs-22-402	+	+	+
13	Rs-22-403	+	+	+
14	Rs-22-405	+	+	+
15	Rs-22-406	+	+	+
16	Rs-23-408	+	+	+
17	Rs-23-411	+	+	+
18	Rs-23-414	+	+	+
19	Rs-23-416	+	+	+
20	Rs-23-417	+	+	+
21	Rs-23-418	+	+	+
22	Rs-24-419	+	+	+

GENETIC CONFIRMATION OF *R. solanacearum* STRAINS

DNA extraction was carried out of all the strains of *R. solanacearum* and was used for PCR analysis for conformation of *R. solanacearum* using universal primers 759/760 which amplifies a specific single 282 bp fragment present in all *R. solanacearum* species complex isolates (fig 3.10). Phylotype specific multiplex PCR amplification was also carried out which revealed that all the isolates used in this study showed 144bp phylotype I specific amplicon indicating all the *R. solanacearum* isolates are Phylotype I belonging to Asian origin and an expected 280 bp *R. solanacearum* species complex specific amplicon (fig 3.11).

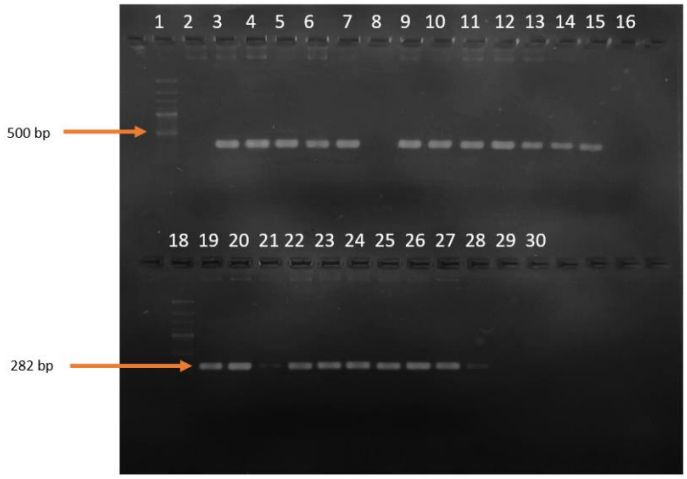

Fig 3.10: PCR analysis for confirmation of *R. solanacearum* isolates.

Lane 1 & 18 represents 1kb DNA Ladder; lane 2- Rs-13-382; lane 3 – Rs-13-383, 282bp ; lane 4- Rs-13-384, 282 bp ; lane 5 – Rs-13-385, 282bp ; lane 6 – Rs-13-386, 282 bp; lane 7- Rs-13-387, 282bp; lane 8- Rs-15-391, lane 9- Rs-17-392, 282 bp; lane 10- Rs-22-396, 282 bp; lane 11- Rs-22-398, 282 bp; lane 12 - Rs-22-400, 282 bp; lane13- Rs-22-402, 282 bp; lane 14- Rs-22-403, 282 bp; lane 15 - positive control, 282 bp; lane 16- Negative control; lane 19-Rs-22-405, 282 bp; lane 20- Rs-22-406, 282 bp; lane 21 - Rs-23-408, 282 bp; lane 22 - Rs-23-411, 282 bp; lane 23 - Rs-23-414, 282 bp; lane 24 - Rs-23-416, 282 bp; lane 25 - Rs-23-417, 282

bp; lane 26 - Rs-23-418, 282 bp; lane 27 - Rs-24-419, 282 bp, lane 28 – positive control, 282 bp; lane 29- Negative control.

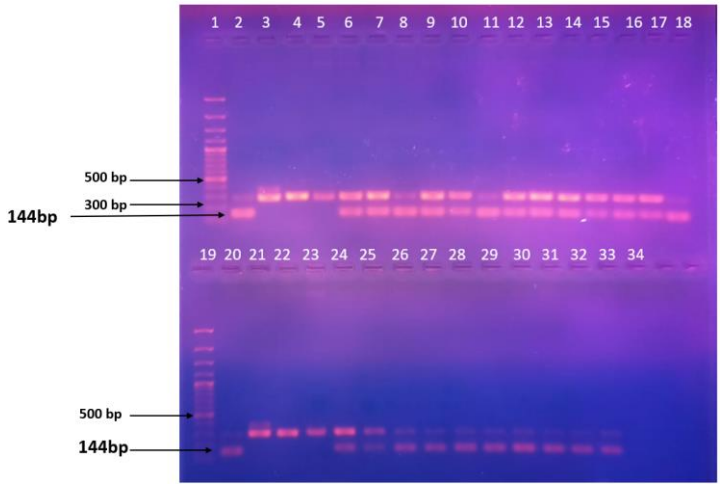

Fig 3.11: Phylotype based classification of *R. solanacearum* isolates.

Lane 1 & 19 represents 100 bp + DNA Ladder; lane 2 & 20- phylotype I, 282 bp, ; lane 3 & 21 – Phylotype 2, 282bp ; lane 4 & 22- Phylotype 3, 282 bp ; lane 5 & 23 – Phylotype 4, 282bp ; lane 6 – Rs-13-383, 282 bp; lane 7- Rs-13-384, 282bp; lane 8- Rs-13-385, 144bp, 282 bp; lane 9- Rs-13-386, 144 bp, 282 bp; lane 10- Rs-13-387, 144 bp, 282 bp; lane 11- Rs-17-392, 144 bp, 282 bp; lane 12 - Rs-22-396, 144 bp, 282 bp; lane13- Rs-22-398, 144 bp, 282 bp; lane 14- Rs-22-400, 144 bp, 282 bp; lane 15 -Rs-22-402, 144 bp, 282 bp; lane 16- Rs-22-403, 144 bp, 282 bp; lane 17 - Rs-22-405, 144 bp, 282 bp; lane 18 – Positive control, 144 bp, 282 bp; lane 24 - Rs-22-406, 144 bp, 282 bp; lane 25 - Rs-23-408, 282 bp; lane 26 - Rs-23-411, 144 bp 282 bp; lane 27 - Rs-23-414, 144 bp 282 bp; lane 28 - Rs-23-416, 144 bp 282 bp; lane 29 – Rs-23-417, 144 bp, 282 bp; lane 30 – Rs-23-418, 144 bp, 282 bp; lane 31- Rs-24-419, 144 bp, 282 bp; lane 32- positive control, 144 bp, 282 bp; lane 33 – Rs-13-382, 144 bp, 282 bp; lane 34- Negative control.

PATHOGENICITY TESTING:

Pathogenicity of isolates were tested on eggplant in greenhouse facility. The disease incidence was recorded in percentage of wilt starting from 3 days after inoculation. Result showed that all the isolates were found to be pathogenic to eggplant, incidence of wilt started vry early in case of isolates can be further divided as highly virulent (Rs-22-396, Rs-23-414, Rs-22-406,

Rs-23-416, Rs-23-417, Rs-23-411,Rs-23-418) and medium virulent (Rs-13-382, Rs-13-383, Rs-13-384, Rs-13-385, Rs-17-392, Rs-22-398, Rs-22-405). 16 isolates out of 22 isolates showed 90% of wilt on day 10thas compared to others like Rs-13-382 isolate showed only 70% wilting observation till the end of the experiment.

Fig 3.12 : Wilting observation of Rs-22-406.

A-Pot 1 showing healthy eggplants(control) and R1, R2 pots showing 40% wilt (day 6 after inoculation) B- Pot 1 showing healthy eggplants (control) and R1, R2 Pots showing 80% wilt (day 9 after inoculation) C- Pot 1 showing healthy eggplants (control) and R1, R2 pots showing 100% wilt (day 13 after inoculation).

Table 3.5: Showing wilting Observation of 6 isolates.

DOI: 5/03/24	No. of Plants		4th DAI	6th DAI	7th DAI	8th DAI	9th DAI	10th DAI	11th DAI	13th DAI	16th DAI	17th DAI
Rs-13-382	5	R1	0	0	0	1	1	3	3	3	5	5
	5	R2	0	0	1	2	2	2	2	3	4	5
Rs-13-383	5	R1	0	2	4	4	5	5	5	5	5	5
	6	R2	0	0	2	2	5	5	5	5	5	5
RS-13-384	6	R1	0	0	2	3	3	4	4	6	6	6
	6	R2	0	1	4	4	4	5	5	5	6	6
Rs-13-385	5	R1	0	0	1	1	1	1	1	1	2	3
	5	R2	0	0	0	3	4	4	4	4	4	5
Rs-17-392	6	R1	0	1	4	4	4	4	4	6	6	6
	6	R2	0	0	1	5	5	5	5	6	6	6
Rs-22-398	5	R1	0	1	2	2	2	2	2	3	4	5
	5	R2	0	1	2	3	5	5	5	5	5	5

Table 3.6: showing wilting observations of 6 isolates

DOI: 6/03/24	No. of Plants		3rd DAI	5th DAI	6th DAI	7th DAI	8th DAI	9th DAI	10th DAI	12th DAI	15th DAI	16th DAI
Rs-22-405	5	R1	0	1	3	4	4	4	4	4	5	5
	5	R2	0	1	2	3	4	4	4	5	5	5
Rs-22-406	5	R1	0	1	1	1	4	5	5	5	5	5
	5	R2	0	1	2	4	4	4	5	5	5	5
Rs-23-416	5	R1	0	0	1	1	4	4	4	5	5	
	6	R2	0	1	3	3	5	5	5	5	6	
Rs-23-417	5	R1	0	1	3	3	4	4	4	4	5	
	5	R2	0	1	3	4	5	5	5	5	5	
Rs-23-418	5	R1	0	2	4	4	5	5	5	5	5	
	5	R2	0	2	4	5	5	5	5	5	5	

Table 3.7: showing wilting observations of 7 isolates

DOI: 7/03/24	No. of Plants		4th DAI	5th DAI	6th DAI	7th DAI	8th DAI	9th DAI	11th DAI	14th DAI
Rs-22-400	5	R1	0	2	2	4	5	5	5	5
	5	R2	0	3	4	4	4	4	4	5
Rs-22-402	5	R1	0	1	1	2	2	3	4	5
	5	R2	0	2	3	4	4	5	5	5
Rs-22-403	5	R1	0	0	0	1	4	4	4	5
	5	R2	0	1	1	2	4	4	5	5
Rs-22-408	5	R1	0	1	1	2	4	4	5	5
	5	R2	0	1	1	2	3	4	4	5
Rs-23-411	6	R1	0	1	1	4	5	5	6	
	6	R2	0	2	3	4	6	6	6	
Rs-23-414	5	R1	0	1	1	3	4	5	5	5
	5	R2	0	1	1	3	3	3	4	5
Rs-22-396	5	R1	0	2	3	3	4	5	5	
	5	R2	0	2	2	3	3	4	5	

CHAPTER 4: ANALYSIS AND CONCLUSION

In this study, a total of 22 *R. solanacerum* isolates were obtained from different agricultural fields. All these isolates produced typical *R. solanacearum* type colonies on BG media and were found pathogenic to eggplant when tested. To further confirm the identity of the organism, PCR amplification using *R. solanacearum* species specific primer pair 759/760 (Opina et al, 1997) was carried out which showed a single 280 bp fragment thus confirming the organism. CWDE of *R. solanacearum* are known to contribute to bacterial wilt (Hayward, 2000). All the isolates used in this study were found to be positive for CWDE mainly cellulase and pectinase. Liu H., (2005) reported that it is not fully known whether *R. solanacearum* with a defective T2SS is weakly virulent which can be due to absence of all the CWDE or the loss of other secreted proteins that contribute to disease. Previous findings suggested that wounded stem infection found that the major factor in causing wilt symptoms was the high-molecular-mass acidic extracellular polysaccharide (EPS I), but the β-1,4-endoglucanase (EG) also contributes to virulence (Saile E et al, 1997). *R. solanacearum* exhibits twitching motility over solid surfaces and this form of motility consists of a flagella-independent translocation requiring retractile type IV pili (fimbriae), which is present on *R. solanacearum* which makes it being related to twitching motility, adherence to surfaces and natural transformation which suggests that twitching also plays a role in pathogenesis of *R. solanacearum.* In this study all the isolates were found to be positive for EPS production and also for twitching motility indicating that it can be one of the factors for causing bacterial wilt.

Biovar determination indicated that all the isolates belong to biovar 3 similarly Kumar et al, (2013) who also reported that *R. solanacearum* isolates from India infecting edible ginger and other solanaceous crops were belongs to biovar 3 and Phylotype I. These isolates were further classified based on their phylotype classification using Phylotype specific multiplex PCR

amplification which revealed that all the isolates used in this study showed 144bp phylotype I specific amplicon indicating all the *R. solanacearum* isolates are Phylotype I belonging to Asian origin, the results obtained are consistent with past research work regarding *R. solanacearum*, which states that all *R. solanacearum* isolates from Asia are phylotype I (Fegan and Prior 2005). Castillo and Greenberg, 2007 stated that there is genetic evidence that phylotypes emerged from geographical isolation and based on the fixed polymorphisms observed among phylotypes one can say that populations may have diverged long time ago. All the isolates were pathogenic on eggplant when inoculated in greenhouse experiment. Further the results showed that *R. solanacearum* showed varying degrees of virulence as some isolates were highly virulent and some were weakly virulent on eggplant. Rs-22-405 isolate showed around 20% wilt on day 5 after inoculation are progressed to 80% on day 13 while isolate Rs-23-411 showed 92.3% wilt on day 9 after inoculation.

CONCLUSION

The study involved 22 isolates, 21 were obtained from ICAR CCARI, Goa and 1 was isolated in Goa University. To further confirm the identity of the organism, PCR amplification using *R. solanacearum* species specific primer pair 759/760 was carried out which showed a single 282 bp fragment thus confirming the organism. The study involved characterization of virulence factors- EPS production, twitching motility, Cell wall degrading enzymes namely pectinase and cellulase. All the isolates were found to be positive for virulence factors. Biovar determination indicated that all the isolates belong to biovar 3. Further, Multiplex PCR was carried out for phylotype classification using Phylotype specific multiplex PCR amplification which revealed that all the isolates used in this study showed 144bp phylotype I specific amplicon indicating all the *R. solanacearum* isolates are Phylotype I. Pathogenicity was tested on eggplant in glasshouse Facility which revealed that *R. solanacearum* showed varying degrees of virulence as some isolates were highly virulent and some were weakly virulent on eggplant. Rs-22-405 isolate showed around 20% wilt on day 5 after inoculation are progressed to 80% on day 13 while isolate Rs-23-411 showed 92.3% wilt on day 9 after inoculation. This study aims to help in finding out the diversity in pathogenicity of R. solanacearum. Since R. solanacearum is a deadly pathogen there is need to develop appropriate biocontrol strategy to control the disease in open condition thereby helping the farmers improve yield of the crop.

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Appendix I

BG medium

Peptone	10.0 g
Yeast Extract	1.0 g
Tryptone	1.0 g
Glucose	5.0 g
DW	1000 mL
Agar	15 g

TZC

TZC agar: One mL of 1% tetrazolium chloride was added per liter BG agar to make one litre TZC agar. 1% stock of TZC was prepared by dissolving 1.0 g TZC in 100 mL DW and filter sterilized.

BG Broth

Peptone	10.0 g
Yeast Extract	1.0 g
Tryptone	1.0 g
Glucose	5.0 g
DW	1000 mL

Pectin agar medium

Citrus pectin	5.0 g
Yeast extract	5.0 g
Agar	15.0 g
pH	7.0
Distilled water	1000 mL

Carboxy Methyl Cellulose (CMC) medium

CMC	10.0 g
NaNO3	1.0 g
K2HPO4	1.0 g
KCl	1.0 g
MgSO4. 7H2O	0.5 g
Yeast extract	0.5 g
Glucose	1.0 g
Agar	15.0 g
Distilled water	1000ml

Basal media

Nh4H2PO4	1 g
KCl	0.2 g
MgSO4.7H2O	0.2 g
Peptone	1 g

Bromothymol blue	0.03 g
Agar	3 g
D.W	1000 ml
pH	7.0-7.1

APPENDIX -II

(REAGENTS)

1% Congo red solution:

composition	Grams
Congo red	1
Distilled water	100

1M NaCl solution:

composition	Grams
NaCl	5.844
Distilled water	100

Buffers and Reagents

50 X Tris acetate EDTA (TAE) Buffer	242.0 g of Tris Base 57.1 mL of Glacial Acetic acid 100 mL 0.5 M EDTA, pH 8.0 Make up the volume to 1000 mL.

0.5 M EDTA pH 8.0	146.12 g EDTA 600 mL D.W. Adjust pH to 8.0 with NaOH Make up the volume to 1000 mL.

1 M Tris-HCl pH 8.0	121.1 g of Tris base 600 mL D.W. Adjust pH to 8.0 with HCl Make up the volume to 1000 mL.

5M NaCl	14.6 g of sodium chloride Make up the volume to 50 mL.

CTAB/NaCl solution	2.05 g of NaCl to 40 ml D.W. Add 5.0 g CTAB, dissolve by heating and stirring Make up the volume to 50 mL.

10 X TE buffer	Add 15.759g of Tris-Cl to 800 ml DW Add 2.92g of EDTA Make the vol to 1 L with DW

10% SDS	10g of SDS in 10ml DW