Research Article Dwivedi & Chouhan, 10(4): April, 2019:6189-6197]
CODEN (USA): IJPLCP **ISSN: 0976-7126**

INTERNATIONALJOURNALOFPHARMACY&LIFESCIENCES
(Int. J. of Pharm. Life Sci.)

Genetic diversity of *Ralstonia solanacearum* causing bacterial wilt of brinjal

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Abstract

In the present study, isolates were identified as *R. solanacearum* on the basis of phenotypic characters as well as based on PCR amplification. All the virulent *R. solanacearum* from Occurrence of bacterial wilt in *solanaceous* crops were noted in 8 states of India as: (ICAR-Res. complex NEH Region). morphologically resembled those from other regions of the world (Williamson *et al*., 2002) All isolates of *R. solanacearum* were collected isolated from Himachal Pradesh (1 isolates), Jharkhand (4 isolates), Uttarakhand (8 isolates), Odisha (9 isolates), IIHR Bangalore (3 isolates), Shillong (2 isolates), Karnataka (3 isolates) and West Bengal (11 isolates). And producing typical white coloured fluidal colonies with pink centres and irregular in shape (Hayward, 1964). The disease affects brinjal crops. Maximum incidence of disease was found in rainy season from July to October up to 60% where as in summer season April to June up to 10%., however, the disease incidence was found up to 60% in Solan districts in Himachal Pradesh and Nainital (mostly foothills area, Golapar) in Uttarakhand. Brinjal crop was moderately infected by *R. solanacearum* and disease incidence was varied 15 - 40% in Palampur area in Himachal Pradesh, 4- 15 % in Plandu district of Ranchi area in Jharkhand. It has been reported Characterization of bacteria degrading 3-hydroxy palmitic acid metylester (3OH-PAME), a quorum sensing molecule of *Ralstonia solanacerum Letters*.And It was observed that the Studies on *Pseudomonas solanacearum* (E.F. Smith) causing wilt of brinjal, in Mysore state.

Key- words: Gene, diversity, phenotype

Introduction

Brinjal or eggplant (Solanum melongena L.*)* is recognized as one of the most important members of the *Solanaceae* family which includes economically important species like potato, tomato, tobacco and pepper (Doganlar *et al*., 2002*b;* Knapp *et al.*, 2013). Eggplant is grown extensively as cash crop by mostly small-scale farmers in many countries. Eggplant (Solanum melongena L.) is an important vegetable in central, southern and south-east Asia, and in a number of African countries. Together with China and India, the Philippines is one of the top 10 eggplant-producing countries in the world based on area of production (FAO Crop Stat, 2012). Several studies report approaches to minimize losses and maintain nutritional value in fruits and vegetables (Ozkaya andDündar, 2009a, b). Eggplant is grown on 1,957,603 hectares, with a total production of 32,699,078 tonnes (FAO, 2008). As the fourth leading eggplant producer after China, India and Egypt, Turkey is an important eggplant producer with an annual production of 813,686 tones (FAO, 2008).

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In 2013, global production of eggplants was 49.4 million tonnes, with 57% of output coming from China alone. India (27% of world total), Iran, Egypt and Turkey were also major producers which, when combined with other Asian countries, constituted 94% of world production. More than 1,600,000 hectares (4,000,000 acres) are devoted to the cultivation of eggplants in the world.

Nutritive value of Brinjal

Nutritive value of Brinjal crops is very important in iron, calcium and other minerals in eggplant supply the essential nutrients required by the body.

Table 1: Nutrition value of brnjal

Constituents	**Nutritive value (100 g of Brinjal**
Amount Per 100 grams	
Calories 25	**Total Fat 0.2 g**
Saturated fat 0 g	0%
Polyunsaturated fat 0.1 g	0%
Monounsaturated fat 0 g	0%
Cholesterol 0 mg	0%
Sodium 2 mg	0%
Potassium 229 mg	6%
Total Carbohydrate 6 g	2%

Dietary fiber 3 g	12%
Sugar 3.5 g	
Protein 1 g	2%
Vitamin A	0%
Calcium	0%

Diseases: Among those diseases, bacterial diseases are *viz*; bacterial cancer, bacterial speck, bacterial spot, bacterial wilt, damping off, *Verticillium* wilt, are found in India and world.

Table 2: Some diseases in brinjal

Name of Disease	Causal Organism	Host
A Fungal diseases		
Damping off	*Phythium aphanidermatum, P. arrhenomanes,P.debaryanum, P.myriotylum , fusarium oxyporum, phomopsis vexans.*	Eggplant
Rhizoctonia damping off and Fruit rot	*Rhizoctoniasolani* (Teleomorph:*Thanatephorus cucumeris)*	Eggplant
Verticillium wilt	*Verticillium albo-atrum, V.dahliae*	Eggplant
White mould/Sclerotinia blight	*Sclerotinia sclerotirum,S.minor*	Eggplant
Buckeye fruits and root rot	*Phytophthora capsici, p. nicotianae Var. parasitica, p. parasitica*	Eggplant
Bacterial diseases		
Bacterial wilt	*Ralstonia solanacerum*	Eggplant
Mycoplasmaldiseases		
Little Leaf of Brinjal	*Phytoplasma*	Eggplant

Genetic Diversity of *R. solanacearum*

Recently, techniques such as multilocus sequence typing (MLST) sequencing (Castillo &Greenber*g*, 2007) or comparative genomic hybridization (CGH) (Guidot *et al*., 2007) were used to investigate the genetic diversity of *R. solanacearum* strains. Members of *R. solanacearum* comprise a relatively diverse group of isolates referred to as a species complex (Gillings and Fahy, 1994) and is classified into 5 races (Buddenhagen *et al*., 1962) based on the host range: Race 1 (*Solanaceous vegetables*), Race 2 (banana), Race 3 (potato and tomato from temperate regions), Race 4 (ginger), Race 5 (mulberry) and 6 biovars (X*ue et al*., 2011), 4 phylotypes based on the ITS region, *hrpB* gene and *flic* gene sequences (Fegan and Prior, 2005). Bacterial wilt affects mainly the *solanaceous* vegetables in India (Singh et al., 1997). Khan *et al*., (1974) reported Asiaticum group of *R. solanacearum* from India on the basis of comparative studies of the isolate infecting solanaceous crops.

Material and Methods

Sample collection and isolation of bacteria from soil

The bacterium *Ralstonia solanacearum* was isolated from infected brinjal (*solanummelongena*) by standard casamino acid pepton glucose (CPG) agar medium (Kelman 1954).The rhizospheric soil of plant sample were taken from the 8 different states: of India: Himachal Pradesh, Jharkhand, IIHR Bangalore, Uttarakhand, Odisha, West Bengal, Shillong and Karnataka (ICAR-Res. complex NEH Region).and the sample carried to the Laboratory, Division of Plant Pathology, IARI, New Delhi for screening of soil bacteria.

Purification of the selected colony:

After selecting the right type of colonies, transferred them on to the CPG slants. Touch wire loop of the inoculation needle on a well-isolated colony and streaked it on the agar slant in a tube, the cultures obtained from singly colony needs to be checked for purity. Makes a dilute suspension of the culture in water and streaked on the CPG agar plates. Culture was pure only one type of colonies was seined**.**

Media and dyes used for study of bacterial samples.

Table 3: Composition of CPG Agar Media

Peptone	10.0 gm
Casein Acid hydrosylate	1.0 gm
Glucose	10.0 gm
Distilled water	1000 ml
Agar	16.0 gm

Table 4: Composition of CPG Broth

Peptone	10.0 gm
Casein Acid hydrosylate	1.0 gm
Glucose	10.0 gm
Distilled water	1000 ml
Ph	7.0

Electrophoresis Reagent:

Table 5: 50X TAE

Tris base	242.0g
Glacial acetic acid	57.1m
O.5M EDTA (pH-8)	100ml
Distilled water:	1000ml

Mix tris with stir bar to dissolve in about 600ml of double distilled water. Add the EDTA and Acetic acid. Bring final volume to 1l with double distilled water. Stored at room temperature.

Table 6: Loading dye

1%Bromophenol blue	200µl
Glycerol	200µl
10% SDS	60µl
0.5M EDTA	50µ
10XTAE	60µ
Distilled water	30µl

Table 7: EDTA (0.5M) (M.W.-372.24)

EDTA	46.52gm
Distilled water	250ml
pH	8.0

Dissolve EDTA in 250ml D/W. Adjust pH with NaOH pellets.

Table 8: TE (10mM)

1M Tris	0.5ml
0.5M EDTA	0.1ml
Distilled water	50ml

Table 9: DNAse 1 buffer

50mM Tris	2.85gm
1mM MgCl2	0.2gm
Distilled water	100ml

Table 10: 10% SDS

SDS	10gm
Distilled water	90ml

Table 11: CTAB /NaCl

NaCl	4.1gm
CTAB	10gm
Distilled water	80ml

Table 12: Composition of SMSA Media

Peptone	10.0g
Glycerol	5ml
casamino acid	1g
Agar	15g
Distilled water	1000ml),
Bacitracin	25mg
Polymyxin ß sulfate	100mg
Chloramphenicol	5mg
Penicillin G	0.5mg
Crystal violet	5mg
TTC	50mg

Quantification of DNA

By Gel electrophoresis method

Cast the agrose gel (0.5 %) in 50 x TAE buffer adding ethidium bromide just before pouring. After solidification load the 5 µl of DNA with dye, and run the gel at 70 V for 6 hrs. View the gel under UV.

Quantification of bacterial DNA by Nanodrop:-

After the isolation of genomic DNA, it was quantified by Nanodrop spectrophotometer. 1ul of DNA was used to analyze in nanodrop and the quantity of DNA (ng/µl) was recorded. For knowing the purity of DNA sample. OD values were recorded at 260 and 280 nm.

Identification of phylotyping of *Ralstonia solanacerum*

38 isolates of *Ralstonia solanacearum* isolated from Brinjal crops from states of Uttarakand, Jharkhand, Odisha, Himachal Pradesh, West Bengal, Shillong, Bangalore, and Karnataka. Phylotype affiliation of each isolates was determined as described (fragan and prior, 2005: fegan 2005).

Table 12: Phylotyping (Multiplex-PCR) Reaction

PCR reaction mixture	**Volume / reaction**
5X PCR buffer	5.0µl
25mM $MgCl_2$	1.5 µl
10mM dNTPs	0.5 µl
Primer Nmult:2:lnF	1 µl
Primer Nmult:22:lnR	1 µl
Nmult:23:AF	1 µl
OLl1	1 µl
Taq polymerase	0.25 µl
100ng DNA Template	1.0 µl
Nuclease free water	18.25 µl
Total Volume	**25.0 µl**

The following Cycling program was used in the Master cycler Gradient -

Initial denaturation	96'c for	5"
	30 Cycles	
Anneling	59'c	30'
Extension	72'c	30'
Final extension	72'c	10

A 10 µl aliquot of each primer amplified product was subjected to electrophoresis on 1.5% agarose gel, stain with ethidium bromide and visualized as phylotype 1which is produce 2 bands: 144bp & 288bp.The 288bp band amplified by 16s rRNA primer using in master mix, that sequence conserverd in all *R. solanacerum* bacteria.

Table 13: The set of Oligonucleotide primers used for Multiplex PCR of *Ralstonia solanacearum*:

Primer designation	Sequences of primers (5-3')	Amplicon size
OL-1-F	ACTAACGAAGCAGAGATGCATTA	144bp
OL-1-R	CCCAGTCACGGCAGAGACT	
Y2-5-F	AACTTAAAGGAATTGACGGAAG	288bp
Y2-6-R	GCATCACAGACCTGTTATTGCCTC	

Results and Discussion

Table 14: Sample collection and isolation of bacteria from soil

S. No.	Isolate	Geographical Region in India	Host	Biovar	Race	Phylotype
1.	UTB-1	Niglat, Almora, Uttarakhand	Brinjal	3	1	1
2.	UTB-2	NBPGR, Bhuali, Uttarakhand	Brinjal	3	1	1
3.	UTB-3	Tharali, Almora, Uttarakhand	Brinjal	3	1	1
4.	UTB-4	Mehra, Almora, Uttarakhand	Brinjal	3	1	1
5.	UTB-5	Ghorakhal. Almora, Uttarakhand	Brinjal	3	1	1
6.	UTB-6	Lakhani, Almora, Uttarakhand	Brinjal	3	1	1
7.	UTB-7	Machhlidibbi, Almora, Uttarakhand	Brinjal	3	1	1
8.	UTB-8	Shamkhat, Almora, Uttarakhand	Brinjal	3	1	1
9.	HPB-14	Palampur, Himanchal Pradesh	Brinjal	3	1	1
10.	JHB-1	Madnadih, Jaamtara, Jharkhand	Brinjal	3	1	
11.	JHB-6	Tangibandh, Deoghar, Jharkhand	Brinjal	3	1	1
12.	JHB-10	Plandu, Ranchi, Jharkhand	Brinjal	3	1	1
13.	JHB-14	Plandu, Ranchi, Jharkhand	Brinjal	3	1	1
14.	ORB-1	Bankala, Sambalpur, Odisha	Brinjal	3	1	1
15.	ORB-2	OAUT, Bhuvneshwer, Odisha	Brinjal	3	1	1
16.	ORB-3	OAUT, Bhuvneshwer, Odisha	Brinjal	3	1	1
17.	ORB-4	Jagatpur, Odisha	Brinjal	3	1	1

18.	ORB-5	Jagatpur, Odisha	Brinjal	3	1	1
19.	ORB-6	Ohinipur, Cuttck, Odisha	Brinjal	3	1	1
20.	ORB-7	Ohinipur, Cuttck, Odisha	Brinjal	3	1	1
21.	ORB-8	Bhuvneshwer, Odisha	Brinjal	3	1	1
22.	ORB-9	Bhuvneshwer, Odisha	Brinjal	3	1	1
23.	WBB-1	Kalyali, Mohanpur, West Bengal	Brinjal	3	1	1
24.	WBB-2	Nimtara, Mohanpur, West Bengal	Brinjal	3	1	1
25.	WBB-3	B.C.K.B, Mohanpur, West Bengal	Brinjal	3	1	1
26.	WBB-4	Haripur, Mohanpur, West Bengal	Brinjal	3	1	1
27.	WBB-5	Binuria, Sriniketan, West Bengal	Brinjal	3	1	1
28.	WBB-6	Lalgarh ,Sriniketan, West Bengal	Brinjal	3	1	1
29.	WBB-7	Dhawali, Sriniketan, West Bengal	Brinjal	3	1	1
30.	WBB-8	Raipur, Sriniketan, West Bengal	Brinjal	3	1	1
31.	WBB-9	Raipur, Sriniketan, West Bengal	Brinjal	3	1	1
32.	WBB-10	Mirzapur, Ohanpur, West Bengal	Brinjal	3	1	1
33.	WBB-11	Madhupur, Pundobari, West Bengal	Brinjal	3	1	1
34.	BRS-57	IIHR, Bangalore	Brinjal	3	1	1
35.	BRS-58	IIHR, Bangalore	Brinjal	3	1	1
36.	BRS-59	IIHR, Bangalore	Brinjal	3	1	1
37.	SBR-1	Shillong	Brinjal	3	1	1
38.	SBR-2	Shillong	Brinjal	3	1	1

Biovar characterization:-

Our 38 isolates *R. solanacearum* of belong to biovar-3, on the basis of identified by utilization of disaccharides and hexose alcohols. The result of the biovar test showed that all out of 38 isolates are already done and 2 groups of *R. solanacearum* isolates (SBR-1, SBR-2) are done by kit protocol. oxidized disaccharides (Sucrose, lactose, maltose; numbers of wells -1, 3, 9) and sugar alcohols (manitol, sorbitol and dulcitol; numbers of wells -20, 19, 17) within 3-5 days. The oxidation reaction was indicated by the change of color. The results revealed a change of color blue to yellow color indicating the oxidization of sugars by bacterial isolates. Therefore, all isolates of *R. solanacearum* isolates belong to biovar III.

Table 15: Pattern of carbohydrate utilization or acid production for each of 5 biovar of *Ralstonia solanacearum*.

Test Utilization of	Biovar 1	Biovar 2	Biovar 2+	Biovar 3	Biovar 4	Biovar 5
Maltose	_	+	+	+	_	+
Lactose	_	+	_	+	_	+
Culobiose	_	+	_	+		+
Mannitol	_	+	_	+	+	+
Sorbitol	_	+	_	+	+	_
Dulcitol	_	+	_	+	+	_
Dextrose	+	+	+	+	+	+
Trehalose	+	_	+	+	+	+
Oxidation of						
Lactose	_	+	+	+	_	+
Maltose	_	+	+	+	_	+
D(+)Cellobiose	_	+	+	+	_	+

Table 16: Classification of the strains based on molecular technique analysis and sequence analysis of various regions of the genome (Fegan and prior, 2005a)

Genotype Groups	*Strain/biovars*	*Geographical Origin*
Phylotype I	All strains of biovar 3, 4and 5	Asia
Phylotype II	All race 3 potato 1, 2 & 2T (Sub Group of biovar2)	America
	Race 2 Banana biovar 1,2 & 2T (Sub group of biovar2)	America
Phylotype III	Biovar1 & 2T	Africa, Surrounding Island
Phylotype IV	More heterogeneous with Biovar 1, 2 and 2T	Indonesia, Australia, Japan

Phylotype:

Based on multiplex-PCR analysis, Brinjal out of 18 strains belonged to one phylotype (I). Some reference *R. solanacerum* strains belonged to phylotype I, II, and IV, but phylotype II and III were not detected. Both phylotype I and IV strains were widely distributed from 38 strains. Phylotype I strains comprised biovar 3 strains; Phylotype IV, however, included biovar N2 strains.

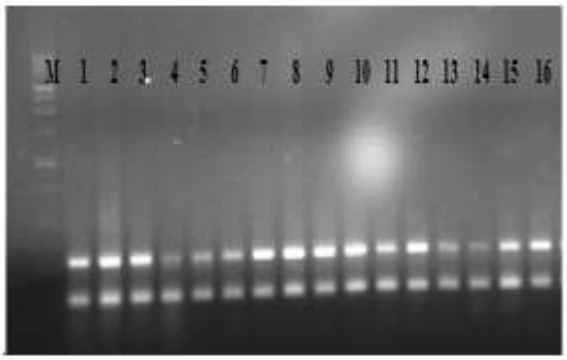
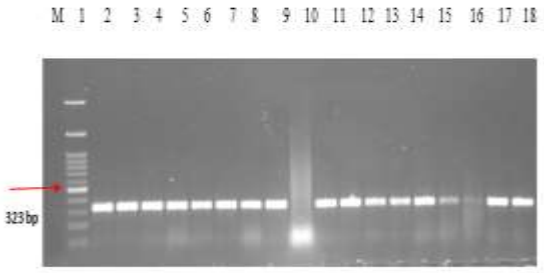


Fig. 4.3: Phylotype of *R. solanacerum* isolated from Brinjal crop showing PCR product of 288bp (i.e. *R. solanacerum*) amplicons for all isolates 144bp (phylotype-1), Lane M =100bp DNA ladder, Lane 1-3: UTB-1, UTB-2 UTB-3 (Uttarakhand), Lanes 4-6: JHB-1, JHB-6, JHB-10 (Jharkhand), lane 7: HPB-14 (Himanchal Pradesh), lanes 8-10:WBB-1, WBB-2, WBB-3 (West Bengal), lanes 11-13: ORB-2, ORB-6, ORB-8, (Orissa), lanes 14-16: BRS-57, BRS-58, BRS-59 (IIHR Bangalore), lanes 17-18: SBR-1, SBR-2 (Shillong isolates).

***Hrp* B gene sequence analysis**

*Hrp*B gene is responsible for hypersensitive reaction and pathogenicity gene located at mega plasmid. 18 isolates of *R. solanacearum* representing biovar were determined and compared to the published sequence of reference strains like- *R. solanacearum* Y45, *R. solanacearum* str. CMR15, *R. solanacearum* Po82, *R. solanacearum* IPO1609 genome, *R. solanacearum* GMI 1000, *R. solanacearum* CFBP2957, *R. solanacearum* DNA for *hrp* gene locus, *R. solanacearumsyzgyii* R24, *R. solanacearum* PSI07, these are the reference genes related to show the similarity coefficient. These reference gene show the Phylotype I, II and IV and biovar 2, 3 and races 1, 2.



Conclusion

All the virulent *R. solanacearum* from Occurrence of bacterial wilt in *solanaceous* crops were noted in 8 states of India as: (ICAR-Res. complex NEH Region). Morphologically resembled those from other regions of the world (Williamson *et al*., 2002) All isolates of *R. solanacearum* were collected isolated from Himachal Pradesh (1 isolates), Jharkhand (4 isolates), Uttarakhand (8 isolates), Odisha (9 isolates), IIHR Bangalore (3 isolates), Shillong (2 isolates), Karnataka (3 isolates) and West Bengal (11 isolates). And producing typical white coloured fluidal colonies with pink centres and irregular in shape (Hayward, 1964). The disease affects brinjal crops. Maximum incidence of disease was found in rainy season from July to October up to 60% where as in summer season April to June up to 10%., however, the disease incidence was found up to 60% in Solan districts in Himachal Pradesh and Nainital (mostly foothills area, Golapar) in Uttarakhand. Brinjal crop was moderately infected by *R. solanacearum* and disease incidence was varied 15 - 40% in Palampur area in Himachal Pradesh, 4- 15 % in Plandu district of Ranchi area in Jharkhand. It has been reported Characterization of bacteria degrading 3-hydroxy palmitic acid metylester (3OH-PAME), a quorum sensing molecule of *Ralstonia solanacerum Letters*(Achari G. A.and Ramesh, R. 2015).And It was observed that the Studies on *Pseudomonas solanacearum* (E.F. Smith) causing wilt of Brinjal, in Mysore state (Khanana 1974).

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How to cite this article

Dwivedi V. and Chauhan U.K. (2019). Genetic diversity of *Ralstonia solanacearum* causing bacterial wilt of brinjal , *Int. J. Pharm. Life Sci.*, 10(4):6189-6197.

Source of Support: Nil; Conflict of Interest: None declared

Received: 01.03.19; Revised: 18.03.19; Accepted: 23.04.19