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Occurrence of Biovars, Races and Phylotyping of *Ralstonia solanacearum* Causing Brown Rot Disease of Potato under Different Agro-climatic Conditions

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Seventy isolates of *Ralstonia solanacearum* were isolated from wilted potato plants and tubers, collected from Uttarakhand, Meghalaya, West Bengal, Himachal Pradesh, Odisha and Karnataka states of India to characterize biovars (bv), races, and phylotypes. The race identification of *R. solanacearum* isolates was done by using differential host such as potato (cv. Kufri Jyoti), tomato (cv. Pusa Ruby) and tobacco (cv. White Burley). Two races were found in potato and race 1 was found all the states except Meghalaya, while race 3 occurred all the states except Karnataka. Biovar of *R. solanacearum* was characterized using the oxidization of disaccharides and sugar alcohols and out of 70 strains, four types of bv was identified and among them bv 3 (42.86%) was prevalent all the states, whereas bv 2 did not find in the West Bengal and Karnataka. For characterization of phylotyping of *R. solanacearum,* phylotype multiplex- PCR was used and three different types of phylotype were identified and among them phylotype I (54.29%) was prevalent in India followed by phylotype IV (34.29%) and phylotype II. It is concluded that bv3, 2 & 2T, races 1 & 3 and phylotypes I, II & IV are prevalent in potato growing areas in India.

Key words: Biovar, race, phylotype, *Ralstonia solanacearum*, potato, 16S rRNA.

Potato is the third most important food crop in the world as well as in India from human consumption point of view, after rice and wheat. India is the second largest potato producer in the world after China. Contribution of potato in agricultural GDP from unit area of cultivable land is about 4 times higher than rice and 4.5 times higher than wheat. India contributes approximately 7.5 percent of the world's total production. (Vision 2030, CPRI Shimla). Potato is cultivated throughout India with area of 1.86 million ha and production of 42.33 million tons (Agropedia, 2011). However, productivity of potato in India is low due to biotic stress including diseases caused by various group of pathogens and poor management practices. Among these, the brown rot disease caused by *Ralstonia solanacearum* (Smith) Yabuuchi is the most important problem in India.

Ralstonia solanacearum [28,30] causing bacterial wilt or brown rot disease is one of the most devastating pathogen of potato. It has broad host range affecting more than 450 plant species distributed in 54 botanical families, including potatoes in tropical, subtropical and temperate regions of the world [28]. In India, the bacterial wilt / brown rot disease is endemic in west coast from Thiruvananthpuram in Kerala to Khera in Gujarat, Karnataka, western Maharashtra, Madhya Pradesh, Uttarakhand, eastern plains of Assam, Odisha and West Bengal, Chhota Nagpur plateau, Andaman and Nicobar Islands and north eastern states of

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India. *R. solanacearum* is a soil borne pathogen that enters the plant through wounds in root tissues and progressively invades the vascular tissues, leading to partial or complete wilting and, ultimately, plant death [5,10]. The global economic losses of potato due to this disease have been estimated at US$950 million per year [6]. In India, bacterial wilt has become a limiting factor in potato cultivation, and cause losses in yield to the tune of 30 to 70 % [25]. Traditionally, *R. solanacearum* complex species and has been subdivided into five races on the basis of differential host [3] and six biovars on the basis of carbohydrate utilization [11]. Based on this classification, potatoes are known to be affected by two races of *R. solanacearum*, that is, race 3 inducing wilt of potatoes under cool temperate conditions and race 1 damage potato crops under tropical and subtropical conditions [16]. Unlike other phytopathogenic bacteria, race systems of *R. solanacearum* are not based on gene-for-gene interactions *i.e.* different cultivars carrying different R genes. Instead, these are determined based on the pathogenicity of each isolate in different kinds of host plants. Although the biovar and race systems are widely accepted for the classification of *R. solanacearum*, however, there is no definite correlation between biovar and race. Each race transects the biovar and each biovar contains various races. The only positive correlation between the biovar and race systems exists for biovar 2 and race 3 [18]. Recently, a new phylogenetic classification system was proposed by Fegan and Prior [7], consisting of four phylotypes, each further divided into sequevar based on *egl* gene. By using the *R. solanacearum* species – specific primers 759/760 in combination with phylotype-specific primers (Nmult:21:1F, Nmult:21:2F, Nmult:23:AF, Nmult:22:Inf and Nmult:22:RR), species and phylotype affiliation can be simultaneously identified in a single PCR assay, called the phylotype-specific multiplex PCR (Pmx-PCR). The phylotyping scheme adds valuable information about the geographical origin and in some cases the pathogenicity of strains. Information on its pathogen population especially biovar, race and phylotyping are essential to formulate a pathogen-targeted and geographically-targeted integrated management strategy against the disease. Therefore, the present study was undertaken to determine the biovar, race, phylotyping and distribution pattern of *R. solanacearum* strains, collected from different agro-climatic regions such as temperate, subtropical and tropical savannah, as well as tropical wet and dry climate of India, causing brown rot disease of potato to step forward for designing an effective management approach.

MATERIALS AND METHODS

Collection of samples and isolation

A survey was carried out to collect the samples of wilted potato plants from major potato growing areas of India such as Meghalaya, Uttarakhand, Odisha, Himachal Pradesh, West Bengal and Karnataka under temperate, subtropical, tropical savannah as well as tropical wet and dry agro-climatic regions. The collected sample was brought to the laboratory for the isolation of *R. solanacearum*. Isolation of bacteria was done by following standard procedure on triphenyl tetrazolium chloride (TTC) medium as described by Schaad *et al*, [20]. The single colony of bacterium showing fluidal, irregular and creamy white with pink at the centre was picked, and maintained on the CPG slants and stored at 4^{o}C for further use.

Molecular characterization of *R. solanacearum*

Bacterial colonies developing the typical fluidal, irregular and creamy white with pink at the centre, colony was selected and subjected to colony polymerase chain reaction (PCR) using *R. solanacearum* specific primers OLI 1 and Y2 [21]. Isolates confirmed in colony PCR, total DNA of bacteria was extracted by CTAB method [17]. PCR amplification was performed in a volume of 25 ìl containing, 100 ng DNA, 5×taq buffer, 25 mM $MgCl_2$, 0.2 µM each primer, 10 mM dNTP, and 1U Taq DNA polymerase (Promega). Amplification was performed in a BIO-RAD C1000 thermo cycler, with an initial denaturation step at 95°C for 2 min; followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 68°C for 20 s, and extension at 72°C for 30 s; and a final extension step at 72°C for 10 min. The PCR products were resolved using a 1.2% agarose gels stained with ethidium bromide at 0.5 µg/ml, and photographed under UV lighting at gel documentation system (Bio-Rad). Further, sequencing of three strains of *R. solanacearum*, *i.e.* MP-1, ORP-11, and UKP-10 belong to different Phylotype *i.e.* Phylotype IV, Phylotype II, and

Phylotype I was done using PCR product of 16S rDNA respectively. The PCR products were resolved using a 1.2% agarose gel. Sequencing of cleaned PCR product was undertaken after cleaning. Nucleotide sequence similarities were determined using BLAST version 2.2.6 (NCBI databases; http://www.ncbi.nlm.nih.gov/). The partial sequences were aligned with the sequences of 16S rRNA gene of *R. solanacearum* obtained from NCBI Gen Bank database. A phylogenetic tree was constructed using neighbor-joining method by MEGA 5.0 software [26].

Pathogenicity test

The positive bacterial isolates were further confirmed by pathogenicity tests on potato cv. Kufri Jyoti. One month old potato plants were used for pathogenicity test in glass house at 28±2 °C temperature and relative humidity70-80%. Bacterial suspension of *R. solanacearum* strains containing 10^8 CFU/ml was inoculated by stem stab inoculation method [29].

Characterization of races

The races of *R. solanacearum* isolated from potato were determined according to Buddenhagen[3] based on differential host of solanaceous crops such as tomato (cv. Pusa Ruby), potato (cv. Kufri Jyoti) and tobacco (cv. White Burley). The plants were raised in glass house (Temp. 28 - 30°C; R.H. 70 – 80%) at National Phytotron Facility, IARI, New Delhi. 48 hour old culture of *R. solanacearum* was inoculated to one month old plants as described by Winstead and Kelman, [29]. For hypersensitivity test, the *R. solanacearum* culture was inoculated on fully expanded leaves of tobacco cv. White Burley by injection into the intracellular space of the leaf with a help of hypodermal syringe [15]. Hypersensitive reaction was observed daily up to fifteen days and wilt symptom was observed at three days intervals.

Determination of biovar

Seventy isolates of *R. solanacearum* were taken, differentiated into biovar based on their ability to utilize disaccharides (sucrose, lactose, maltose) and sugar alcohols (manitol, sorbitol and dulcitol) using KB009 HiCarbohydrate™ Kit (HiMedia Laboratories Pvt. Limited), which contains above mentioned disaccharide and sugar alcohols as described previously Hayward, [9] and He *et al*,[12]. 50 µl of bacterial suspension prepared from 48 hour old culture of *R. solanacearum* strains (0.5 OD at 620 nm) was inoculated into each well by surface inoculation method and incubated at 35±1°C. The observations of changing colour were taken after18h of inoculation of culture.

Phylotype identification

Phylotype identification of the Indian strains of *R. solanacearum* was determined by multiplex PCR using a set of phylotype-specific primers based on *egl* gene [7] and 16S rDNA based primers Y2/OLI 1 (Table1). Amplification was carried out in a total volume of 25 µl containing 5.0 µl of 5X PCR Taq buffer, 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 6 pmoles of the primers Nmult:21:1 F, Nmult:21:2 F, Nmult:22:InF, 18 pmoles of the primer Nmult:23:AF and 4 pmoles of the primers Y2 and OLI 1, 1 unit Taq polymerase and 1 µl of 100 ng DNA templates was used. The following cycling programme was used in a thermal cycler (BIO-RAD C1000 thermo cycler), 96 °C for 5 min and then cycled through 30 cycles of 94 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s, followed by a final extension period of 10 min at 72 °C. A 10 µl aliquot of each amplified PCR product was subjected to electrophoresis and photography as described earlier.

RESULTS

Collection, isolation and characterization of *R. solanacearum* isolates

A total of seventy strains of *R. solanacearum* was isolated from the tuber and stem of wilted potato plant, collected from six states of India such as, Uttarakhand (20), Meghalaya (21), West Bengal (6), Himachal Pradesh (3), Odisha (11), and Karnataka (9) under temperate, subtropical, tropical savanna and tropical wet and dry agro-climatic conditions (Table 2). Colonies of collected strains of *R. solanacearum*, showed virulent, fluidal, irregular and creamy white with pink at the centre on TTC medium. All strains were further confirmed as *R. solanacearum* by colony PCR, using specific primers OLI 1 – Y2, yielding an expected 288-bp fragment of *R. solanacearum* (Fig.1). Further, the sequence analysis of three strains, MP-1 (KP715462), ORP-11(KP715466) and UKP-10 (KP715467), of *R. solanacearum* belonging to Phylotype IV, II and I along with strains of *R. solanacearum* representing different countries based on partial 16S rDNA sequence (288bp) was

done with homology of 95 – 100 %. Based on grouping with the isolates KP715462, KP715466 and KP715467, of *R. solanacearum* were phylogenetically affiliated to the genus *R. solanacearum*, forming a phylogenetic lineage with genus with a bootstrap value of 721. These isolates formed separate 3 cluster. However, KP715467 and KP715466 were closely related to *R. solanacearum* LN681198, *R. solanacearum* KM216391, *R. solanacearum* LN681202, *R. solanacearum* KM085002, *R. solanacearum* KF030881, *R. solanacearum* LN681200, *Ralstonia* sp. KM253164 and KP715462 formed separate cluster (Fig.2). The sequence of KP715462 strains collected from Shillong (Meghalaya) belongs to phylotype IV, formed the separate cluster. The sequence data were submitted to NCBI and obtained accession numbers of *R. solanacearum* isolates MP-1(KP715462), ORP-11(KP715466) and UKP-10 (KP715467).

Pathogenicity test and races identification

The results of pathogenicity test revealed that all the strains of *R. solanacearum* were able to produce wilt symptom on potato plants after 10 days of inoculation. The wilted plants were further confirmed by ooze test. The races of *R. solanacearum* were identifying by pathogenicity test in differential hosts, such as potato (Kufri Jyoti), tomato (Pusa Ruby) and tobacco (White Burley). The pathogenicity test showed that all the strains of *R. solanacearum*, were able to cause wilt symptom in potato and tomato plants, except the strains collected from Meghalaya which did not cause wilt symptom in tomato (Table 2). Those strains, caused wilt symptom in potato, tomato and tobacco were placed under race 1 and those strains fail to produce the wilt symptom in tobacco, only showed chlorosis on the inoculated leaf of tobacco plants after one week of inoculation were placed under race 3. Out of 70 strains of *R. solanacearum*, 51.4% strains belong to race 3 and 48.6% race 1. Race 3 was reported from temperate, subtropical, tropical savannah agro-climatic regions in the states of Meghalaya (100%), Himachal Pradesh (66.66%), Odisha (63.63%), Uttarakhand (20%) and West Bengal (33.33%) except tropical dry and wet climate in Karnataka. Whereas, race 1 dominated in subtropical, tropical savannah and tropical dry and wet climatic condition in the states like Karnataka (100%), Uttarakhand (80%), West Bengal (66.66%), Odisha and Himachal Pradesh, and did not found in the temperate climate in the states of Meghalaya of India.

Biovar determination of *R. solanacearum*

The strains were characterized into biovar on the basis of their ability to utilize disaccharides and to oxidize hexose alcohols (Table 3). It reveals that bv1, bv2, bv2T, and bv3 were present in India to infect potato plant. Out of the 70 strains, 7.14 % belonged to bv1, 28.57 % to bv2, 21.43 % to bv2T and 42.86 % to bv3. The bv1 was found only in Uttarakhand. The bv2 was prevalent in Himachal Pradesh (66.67%), Meghalaya (61.90%), Odisha (27.27%) and Uttarakhand (10%). Bv2T encountered Odisha (36.36%), Meghalaya (33.33%), West Bengal (33.33%) and Uttarakhand (10%). The bv3 prevalent in Karnataka (100%), West Bengal (66.67%), Uttarakhand, Odisha, Himachal Pradesh and Meghalaya. Maximum diversity of biovar (bv1, bv2, bv2T, & bv3) of *R. solanacearum* was recorded in Uttarakhand followed by Meghalaya (bv2, bv2T, & bv3) and Odisha (bv2, bv2T & bv3).

Phylotype identification

Table 1. List of primers used in multiplex PCR

S.N.	Primer Name	Primer sequence	Expected band size	Remarks
1	Nmult:21:F	5'-CGTTGATGAGGCGCGCAATTT-3'	144bp	Phy. I (Asiaticum)
2	Nmult:21:F	5'-AAGTTATGGACGGTGGAAGTC-3'	372bp	Phy. II (Americanum)
3	Nmult:22:InF	5'-ATTGCCAAGACGAGAGAAGTA-3	213bp	Phy. IV (Tropical)
4	Nmult:23:AF	5'-ATTACGAGAGCAATCGAAAGATT-3'	91bp	Phy. III (African)
5	Nmult:22:RR	5'-TCGCTTGACCCTATAACGAGTA-3'		Amorce reverse unique
6	Y2	5'- CCCACTGCTGCCTCCCGTAGGAGT-3'	288bp	*R. solanacearum* Specific primers
7	OLI1	5'GGGGGTAGCTTGCTACCTGCC3'		

Table 2. Characterization of *Ralstonia solanacearum* strains of potato collected from different agro-climatic regions of India

S. No.	Strains	Place of collection	Climatic condition	Year of collection	Source of isolation	16S PCR	Biovar	Phylotype	Pathogenicity reaction on differential host			Race
									Potato (Wilt)	Tomato (Wilt)	Tobacco (Wilt)	
1	UKP 1	Nainital, Uttarakhand	Subtropical	2013	Stem	+	2	IV	+	+	-	3
2	UKP 2	Nainital, Uttarakhand	climate.	2013	Stem	+	2	IV	+	+	-	3
3	UKP 3	Nainital, Uttarakhand	Temp	2013	Tuber	+	3	I	+	+	+	1
4	UKP 4	Nainital, Uttarakhand	.(6^{o}C - 30^{o}C).	2013	Tuber	+	3	I	+	+	+	1
5	UKP 5	Nainital, Uttarakhand	Altitude-	2013	Tuber	+	3	I	+	+	+	1
6	UKP 6	Nainital, Uttarakhand	1654	2013	Tuber	+	1	I	+	+	+	1
7	UKP 7	Nainital, Uttarakhand	meters.	2013	Stem	+	1	I	+	+	+	1
8	UKP 8	Nainital, Uttarakhand		2013	Stem	+	1	I	+	+	+	1
9	UKP 9	Nainital, Uttarakhand		2013	Tuber	+	1	I	+	+	+	1
10	UKP 10	Nainital, Uttarakhand		2013	Tuber	+	1	I	+	+	+	1
11	UKP 11	Nainital, Uttarakhand		2013	Tuber	+	3	I	+	+	+	1
12	UKP 12	Nainital, Uttarakhand		2013	Stem	+	3	I	+	+	+	1
13	UKP 13	Nainital, Uttarakhand		2013	Tuber	+	3	I	+	+	+	1
14	UKP 14	Nainital, Uttarakhand		2013	Stem	+	2T	IV	+	+	-	3
15	UKP 15	Nainital, Uttarakhand		2013	Tuber	+	2T	IV	+	+	-	3
16	UKP 16	Nainital, Uttarakhand		2013	Tuber	+	3	I	+	+	+	1
17	UKP 17	Nainital, Uttarakhand		2013	Tuber	+	3	I	+	+	+	1
18	UKP 18	Nainital, Uttarakhand		2013	Tuber	+	3	I	+	+	+	1
19	UKP 19	Nainital, Uttarakhand		2013	Tuber	+	3	I	+	+	+	1
20	UKP 20	Nainital, Uttarakhand		2013	Tuber	+	3	I	+	+	+	1
21	MP 1	Shillong, Meghalaya	Temperate	2013	Stem	+	2	IV	+	-	-	3
22	MP 2	Shillong, Meghalaya	climate	2013	Stem	+	2T	IV	+	-	-	3
23	MP 3	Shillong, Meghalaya		2013	Stem	+	2	IV	+	-	-	3
24	MP 4	Shillong, Meghalaya		2013	Stem	+	2	IV	+	-	-	3
25	MP 5	Shillong, Meghalaya	Temp.	2013	Stem	+	2	IV	+	-	-	3
26	MP 6	Shillong, Meghalaya	(3^{o}C- 23 ^{o}C).	2013	Stem	+	2	IV	+	-	-	3
27	MP 7	Shillong, Meghalaya	Altitude-	2013	Stem	+	2T	IV	+	-	-	3
28	MP 8	Shillong, Meghalaya	1966 metre.	2013	Tuber	+	2	IV	+	-	-	3
29	MP 9	Shillong, Meghalaya		2013	Stem	+	2	IV	+	-	-	3
30	MP 10	Shillong, Meghalaya		2013	Stem	+	2	IV	+	-	-	3
31	MP 11	Shillong, Meghalaya		2013	Tuber	+	2T	IV	+	-	-	3
32	MP 12	Shillong, Meghalaya		2013	Stem	+	2T	IV	+	-	-	3
33	MP 13	Shillong, Meghalaya		2013	Stem	+	2T	IV	+	-	-	3

34	MP 14	Shillong, Meghalaya		2013	Tuber	+	2	IV	+	-	-	3
35	MP 15	Shillong, Meghalaya		2013	Stem	+	2	IV	+	-	-	3
36	MP 16	Shillong, Meghalaya		2013	Tuber	+	2	IV	+	-	-	3
37	MP 17	Shillong, Meghalaya		2013	Stem	+	2T	IV	+	-	-	3
38	MP 18	Shillong, Meghalaya		2013	Stem	+	2T	IV	+	-	-	3
39	MP 19	Shillong, Meghalaya		2013	Stem	+	2	IV	+	-	-	3
40	MP 20	Shillong, Meghalaya		2013	Stem	+	2	IV	+	-	-	3
41	MP 21	Shillong, Meghalaya		2013	Stem	+	3	I	+	-	-	3
42	WBP 1	Nadia, West Bengal	Tropical	2011	Stem	+	2T	I	+	+	-	3
43	WBP 2	Nadia, West Bengal	savannah.	2011	Stem	+	2T	I	+	+	-	3
44	WBP 3	Nadia, West Bengal	Temp.	2011	Stem	+	3	I	+	+	+	1
45	WBP 4	Nadia, West Bengal	(15°C- 45°C).	2011	Stem	+	3	I	+	+	+	1
46	WBP 5	Nadia, West Bengal		2011	Stem	+	3	I	+	+	+	1
47	WBP 6	Nadia, West Bengal		2011	Stem	+	3	I	+	+	+	1
48	ORP 1	Khurda, Orissa	Tropical	2014	Stem	+	3	I	+	+	+	1
49	ORP 2	Khurda, Orissa	savannah	2014	Stem	+	2T	II	+	+	-	3
50	ORP 3	Khurda, Orissa		2014	Stem	+	3	I	+	+	+	1
51	ORP 4	Bhubneshwar, Orissa		2014	Stem	+	3	I	+	+	+	1
52	ORP 5	Bhubneshwar, Orissa	Temp.(15°C-40°C).	2014	Stem	+	3	I	+	+	+	1
53	ORP 6	Bhubneshwar, Orissa		2014	Tuber	+	2	II	+	+	-	3
54	ORP 7	Bhubneshwar, ,Orissa.		2014	Tuber	+	2	II	+	+	-	3
55	ORP 8	Bhubneshwar ,Orissa		2014	Tuber	+	2	II	+	+	-	3
56	ORP 9	Bhubneshwar ,Orissa		2014	Tuber	+	2T	II	+	+	-	3
57	ORP 10	Bhubneshwar ,Orissa		2014	Tuber	+	2T	II	+	+	-	3
58	ORP 11	Bhubneshwar ,Orissa		2014	Tuber	+	2T	II	+	+	-	3
59	HPP 2	Shimla, Himachal Pradesh	Subtropical.	2010	Stem	+	2	I	+	+	-	3
60	HPP 10	Shimla, Himachal Pradesh	Temp.(1°C-	2010	Stem	+	2	II	+	+	-	3
61	HPP 53	Shimla, Himachal Pradesh	28°C). Alt-2397 m.	2010	Stem	+	3	I	+	+	+	1
62	KP 1	Hassan, Karnataka	Tropical	2014	Tuber	+	3	I	+	+	+	1
63	KP 2	Hassan, Karnataka	savanna (20-35 °C)	2014	Tuber	+	3	I	+	+	+	1
64	KP 3	Dharwad, Karnataka	Tropical Wet	2014	Stem	+	3	I	+	+	+	1
65	KP 4	Dharwad, Karnataka	&Dry climate	2014	Stem	+	3	I	+	+	+	1
66	KP 5	Dharwad, Karnataka	(15°C-40 °C).	2014	Stem	+	3	I	+	+	+	1
67	KP 6	Dharwad, Karnataka		2014	Stem	+	3	I	+	+	+	1
68	KP 7	Dharwad, Karnataka		2014	Stem	+	3	I	+	+	+	1
69	KP 8	Dharwad, Karnataka		2014	Tuber	+	3	I	+	+	+	1
70	KP 9	Dharwad, Karnataka		2014	Tuber	+	3	I	+	+	+	1

For 16S rRNA PCR (+) = Positive reaction; Pathogenicity test - wilt (+), Chlorosis (-).

Table 3. Biovar characterization of *R. solanacearum* strains collected from different states under different agro climatic regions of India

State	No. of strains	Strains of *R. solanacearum* belong to different biovars (%)			
		bv1	bv2	bv2T	bv3
Uttarakhand	20	25.00	10.00	10.00	55.00
Meghalaya	21	0	61.90	33.33	4.76
West Bengal	6	0	0	33.33	66.67
Himachal Pradesh	3	0	66.67	0	33.33
Odisha	11	0	27.27	36.36	36.36
Karnataka	9	0	0	0	100
Total	70	7.14	28.57	21.43	42.86

T – Test value (probability level at 5%): Biovar 1 vs. biovar 2 (0.087), Biovar 1 vs. biovar 2T (0.099), Biovar 1 vs. biovar 3 (0.002), Biovar 2 vs. biovar 2T (0.0752), Biovar 2 vs. biovar 3 (0.200) & Biovar 2T vs. biovar 3 (0.078).

Table 4. Phylotype characterization of *R. solanacearum* collected from different states under different agro climatic regions of India

State	No. of strains	Strains of *R. solanacearum* belong to different phylotype (%)		
		PhylotypeI	PhylotypeII	PhylotypeIV
Uttarakhand	20	80	0	20
Meghalaya	21	4.76	0	95.24
West Bengal	6	100	0	0
Himachal Pradesh	3	66.67	33.33	0
Odisha	11	36.36	63.64	0
Karnataka	9	100	0	0
Total	70	54.29	11.43	34.29

T – Test value (probability level at 5%): Phylotype I vs Phylotype II (0.021), Phylotype I vs Phylotype IV (0.030) & Phylotype II vs Phylotype IV (0.972).

Phylotype multiplex – polymerase chain reaction (Pmx-PCR) revealed that out of four three phylotypes *viz*. phylotype I (Asian), phylotype II (American) and phylotype IV (Tropical) were present in India (Fig. 3). Out of 70 strains, 54.29% belongs to phylotype I, 34.29% phylotype IV and 11.43% phylotype II (Table.4). All the strains of *R. solanacearum*, isolated from West Bengal (tropical savannah) and Karnataka (tropical wet and dry climate) states belong to Phylotype I, whereas 80% strains of *R. solanacearum* from Uttarakhand, 66.67% strains from Himachal Pradesh (both under subtropical climate), 36.36% strains from Odisha (tropical savannah) and 4.76% from Meghalaya (temperate climate) represented Phylotype I. The Phylotype II was found in tropical savannah

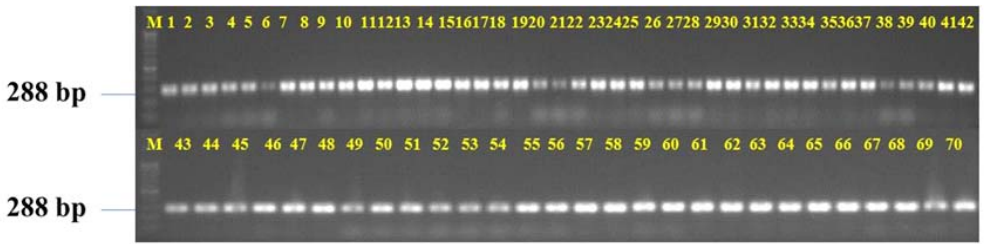

Fig. 1. PCR amplification of *Ralstonia solanacearum* strain at 288 bp using 16S rDNA based primer Y2/OLI 1, Lane M = 100 bp DNA ladder, lanes 1-20 (Uttarakhand), 21-41 (Meghalaya), 42-47 (West Bengal), 48-58 (Odisha), 59-61 (Himachal Pradesh) and 62-70 (Karnataka)

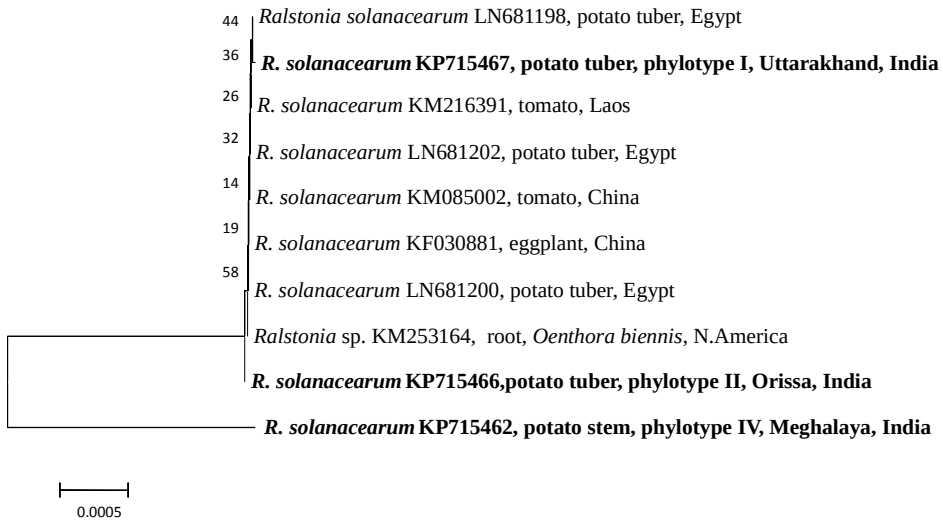

Fig. 2. Phylogeny tree inferred from *R. solanacearum* based on 16S rRNA gene sequences of 3 strains of *R. solanacearum* [MP-1 (KP715462), ORP-11 (KP715466), and UKP-10 (KP715467) isolated from potato along with 7 strains of *R. solanacearum* obtained from NCBI database. The tree was constructed to form a phylogenetic lineage with genus with a bootstrap value of 721 using MEGA 5.0

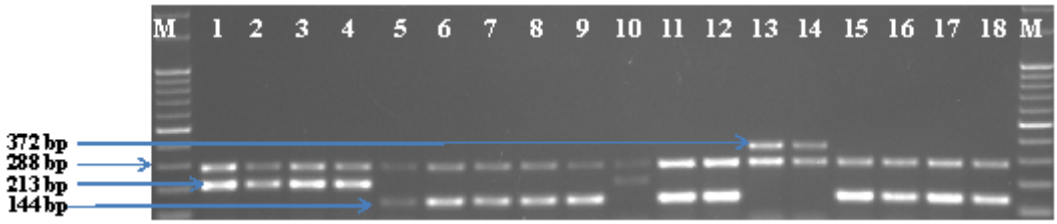

Fig. 3. Phylotype of Indian strain of *R.* solanacearum, isolated from potato, showing PCR products of 288bp (*i.e. R. solanacearum*) amplicons for all isolates, 144bp (phylotype I), 213bp (Phylotype IV) and 372bp (phylotype II) amplicons. Lane M = 100 bp DNA ladder, lanes 1-5 (Meghalaya), 6-10 (Uttarakhand), 11-12 (West Bengal), 13-14 (Orissa), 15-16 (Himachal Pradesh) and 17-18 (Karnataka)

climate, Odisha (63.64%) and subtropical climate, Himachal Pradesh (33.33%) whereas the Phylotype IV was found maximum in temperate climate, Meghalaya (95.24%) followed by subtropical climate, Uttarakhand (20%). But it could not record from tropical climate in the states of Karnataka, West Bengal, Odisha.

DISCUSSION

In the present studies, seventy strains of *R. solanacearum* were isolated from bacterial wilt / brown rot infected potato plants and tubers, collected from potato growing areas of different states such as, Uttarakhand, Meghalaya, West Bengal, Himachal Pradesh, Odisha and Karnataka under temperate, subtropical, tropical savannah and tropical wet & dry agro climatic regions of India. The strains of *R. solanacearum* showed fluidal, irregular and creamy white with pink at the centre on TTC medium and showed positive reaction with biochemical test as described by Schaad *et al*, [20]. The strains of *R. solanacearum* were confirmed by molecular level using a set of specific primer OLI 1 and Y2 [21]. These strains also showed positive pathogenicity test in potato cv. Kufri Jyoti. Further the same bacterial suspension was inoculated on tomato cv. Pusa Ruby and tobacco cv. White Burley for race characterization on the basis of differential host and disease based reaction. The result showed strains of *R. solanacearum*, causing bacterial wilt / brown rot

disease in potato, belong to race 1 and race 3. The findings of the present study are also supported by Buddenhagen *et al*. [3], who classified *R. solanacearum* into three races who found only one race. Race 1 infects many solanaceous plants such as brinjal, tomato, tobacco, pepper and other plants including some weeds. In addition to race 2 that causes wilt of triploid banana (*Musa* spp.) and *Heliconia* spp., while race 3 affects potato and tomato but it is weakly virulent on other solanaceous crops. Aragaki and Quinon [2] reported that race 4 infected ginger in the Philippines. He et al. [12] reported race 5 from mulberry in China. Five races have been described so far, but they differ in host range, geographical distribution and ability to survive under different environmental conditions [8].

In our study, race 3 was found prevalent not only in high hills of Meghalaya, Uttarakhand and Himachal Pradesh, also in plains of Odisha and West Bengal, but did not found in Karnataka. Shekhawat *et al* [23] reported that in India r3bv2 was persist primarily under cool humid conditions in hilly areas, though reported in a few locations in eastern plains and Deccan plateau. Whereas race 1 was found to be prevalent in tropical savannah and tropical dry & wet agro-climatic condition states of West Bengal, Odisha and Karnataka, but it was also found in subtropical climatic condition of Uttarakhand and Himachal Pradesh, which was confirmation to earlier report [14]. The distribution of races in different agro-climatic conditions may be due to the movement of seed potato from plains to hills and vegetable potato from hill to plain, might results in the introduction of race 1 in hills and occurrence of race 3 in states of Odisha and West Bengal under tropical savannah climate.

The result of biovar test demonstrated that the strain of *R. solanacearum* collected from Uttarakhand belong to four biovars i.e. bv1, bv2 , bv2T & bv3, from Meghalaya & Odisha bv2, bv2T & bv3, West Bengal strains belong to bv2T & bv3, Himachal Pradesh strains belong to bv2 & bv3 and Karnataka isolates belong to bv3. Sagar et al, [19], reported Himachal Pradesh isolates belong to biovar 2, 3 & 4, West Bengal isolates belong to biovar 2 & 3 and Meghalaya isolates belong to biovar 2, 2T. These results confirmed our findings. Although all the strains isolated from the potato belong to race 1 & 3. However, races and biovars are poorly correlated except for race 3, which is more or less similar to bv2 [8]. Titatarn, [27] classified the bacterial wilt pathogen of potato as bv3 and bv4 from mid hills and bv2 from high hills of Thailand. Ahmed et al, [1] also reported bv3 belong to race 3, which causes bacterial wilt of potato in Bangladesh. Shekhawat et al, [22] reported race 1 bv3 of *R. solanacearum*, cause of wilt of potato in plain and plateau region of India. Therefore, with this study it is observed that bv1, bv2, bv2T & bv3 of *R. solanacearum* belong to race 1 & 3, causing brown rot disease of potato in different agro-climatic regions of India. Bv1 was found in Uttarkhand. Bv1 is the most widely distributed strain of *R. solanacearum* in the world [12]. Hence it is not surprising that bv1 is among the isolates collected from India as several thousand of potato genotype has been introduced to the country from the different parts of the world to develop high yielding and adaptable cultivar with resistance to major stresses.

The phylotyping results of the *R. solanacearum* collected from different parts of India also reveals that phylotype I dominated in India, including Uttarakhand, Meghalaya, West Bengal, Himachal Pradesh, Odisha and Karnataka followed by phylotype IV and phylotype II. However, phylotype III was not observed in India. Phylotype II, found in Himachal Pradesh and Odisha where as phylotype IV, occurred in Meghalaya and Uttarakhand, which was slightly different to earlier report [19]. Phylotype I strains causing bacterial wilt of potato includes *R. solanacearum* isolates traditionally classified as 3, 4, and 5 are primarily isolated in Asia [7]. Also *R. solanacearum* strain as which cluster into Phylotype I encompasses a majority of low land (tropical) strains with a wide host range[4]. Phylotype II included strains belonging to biovar 1, 2, and 2T isolated primarily from America. Whereas phylotype IV contains strains isolated primarily from Indonesia belonging to biovar 1, 2 and 2T. Phylotype IV strains have been reported from Philippine, Japan, Australia and Indonesia [7], they are known to be widely distributed in Japan [13]. However, in this study, phylotype IV was identified in strains of *R. solanacearum* isolated from potato collected from hills of Meghalaya (temperate climate) and Uttarakhand (subtropical climate) states of India. Wicker

. [28] agreed that the phylotyping scheme proposed by Fegan and Prior [7] was broadly consistent with the former phenotypic and molecular typing schemes and added valuable information about the geographical origin and in some cases the pathogenicity of strains.

CONCLUSION

The incidence of bacterial wilt varied in the major potato growing areas may be due to the species complex of the pathogen, *R. solanacearum* and also for various soil factors. Biovars 1, 2T & 3, race 1 & 3 and Phylotype I, II & IV, of *R. solanacearum* was prevalent in all potato growing areas of hill and plains under different agro-climatic regions of India. Phylotype I prevalent in plains of Karnataka, West Bengal and hill of Uttarakhand and Himachal Pradesh, Phylotype II prevalent in plains of Odisha & hill of Himachal Pradesh and Phylotype IV prevalent in hill of Meghalaya and Uttarakhand states. The findings of the present study will be useful for mapping of population structures of *R. solanacearum* using the molecular approaches with special emphasis on its integrated disease management.

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