International Journal of Advanced Biotechnology and Research(IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-6, Issue-3, 2015, pp290-295
http://www.bipublication.com

Research Article

Repetitive PCR Based Genetic Diversity of *RALSTONIA SOLANACEARAM* Associated With Chilli and Potato from Marathwada Region of Maharashtra

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[Received-01/07/2015, Accepted-06/07/2015, Published-11/07/2015]

ABSTRACT:

Genetic diversity of twenty isolates of *Ralstonia solanacearum* causing bacterial wilt in Chilli (*Capsicum annum*) and Potato (*Solanum tuberosum)* from different locations of Marathwada region Maharashtra was assessed. These 20 isolates were characterized biochemically, on the basis of virulence and hypersensitivity reaction (HR). These 20 strains of *R. solanacearum* isolated from two different host plants were further analyzed by repetitive sequence-based polymerase chain reaction (rep-PCR). The results of rep-PCR confirmed the Race and biovar determined biochemically and host specificity. Though all the strains belonged to Race 1 biovar 3 and race 3 and biovar 2, the PCR analysis segregated in these strains on the basis of their host specificity, race and biovar. These isolates collected from geographical distributed areas of Marathwada were categorized into groups in Rep-PCR on the basis similarity index based on Jaccard coefficient. The repetitive sequence-based polymerase chain reaction (rep-PCR) offers a study method for assessing genetic diversity among the bacterial pathogen that could segregated to Race/ Biovar level.

Keywords: *Ralstonia solanacearum, chilli (Capsicum annum), potato (Solanum tuberosum), TZC medium, bacterial wilt, rep-PCR*

[I] INTRODUCTION

Bacterial wilt is very common and serious disease in plants affecting wide host range. The wilt caused by *R. solanacearum* found in temperate region of the world causes very severe wilt in chilli and potato in tropical region as well [1]. Numerous species of tropical, subtropical and temperate plants are susceptible to races of *R. solanacearum* causing serious losses in several economically important crops [2]. *R. solanacearum* occur in diverse population which differs from the host range, geographical distribution, pathogenicity, genetic

characteristics and physiological properties [3]. The pathogen has been divided in five races and biovar on the basis host range and utilization of sugar alcohols and disaccharides [4]. The description of genetic similarity of the bacterial populations necessitates the study of epidemiology and also for the development of disease management strategies against bacterial wilt. To differentiate the isolates, DNA based methods have been found effective for the analysis of the difference and similarity amongst the isolates, such as restriction fragment length polymorphism (RFLP), 16S rDNA sequence repetitive sequence based polymerase chain reaction (rep- PCR), pulsed-field gel electrophoresis (PFGE) [5].

The Rep-PCR fingerprinting of the isolates makes use of DNA primers to highly conserved repetitive DNA present in number of copies present in the genome of the isolates. The genetic diversity of the *R. solanacearum* in India is not known. The genetic diversity of *R. solanacearum* isolated from potato (*Solanum tuberosum)*, and chilli (*Capsicum annum*), collected from different location of Marathwada District has been carried out and Rep-PCR has successfully shows the occurrence of the diversity in these strains.

[II] MATERIALS AND METHODS

2.1. Isolation and characterization of the pathogen

The isolation of the pathogen was done on the TZC (2, 3, 5 triphenyltetrazolium chloride) medium as described by Kelman [6]. Morphological and biochemical tests were carried out for the characterization of the pathogen, Gram staining, motility, Kovac's Oxidase test, Levan production, Carbohydrate utilization, Arginine production, Aesculin hydrolysis, Gelatin liquification and Tween 80 lypolysis, Tyrosinase activity and Hydrogen peroxide test were also performed as described by Hayward [7].

2.2. DNA isolation and purification

The extraction of genomic DNA was done from the culture grown on YPG medium (Yeast peptone glucose) as method described by Ausubel *et al* [8].

Purity of DNA was checked using Eppendorf Biophotometer. These DNA samples were further used for PCR analysis[9].

2.3 PCR Analysis

The primers as described by Seal *et al* [10] were used for DNA fingerprinting. REP1R-I (5' IIICGICGICATCIGGC 3') and REP2-I (5'ICGITTATCIGGCCTAC 3') PCR amplification of selected isolates was carried out in thermalcycler (Applied Biosystem 9700), as method described by Versalovic *et al.* [11]. Amplified products were separated on 1.5% agarose gel in 1x TAE buffer at 120V and stained with ethidium bromide and visualized on a UV transilluminator[9].

3.4 Data analysis: The gel images of twenty isolates were scored using a binary scoring system, recording the presence and absence of bands as 1 and 0, respectively. The data were exported into a spreadsheet and formatted for the NTSYSpc [12]. Cluster analysis was performed on the similarity matrix using the unweighted pair group method of arithmetic average (UPGMA) the dendogram was analysed by similarity matrix based on jaccard coefficient.

[III] RESULTS

3.1. Isolation and characterization:

A total 20 strains were isolated from the different locations of the Marathwada region on the TZC medium. Spherical shaped isolated colony with variable size, smooth margin and white mucoid with a red center, opaque, motile, gram -ve short rods were observed.

3.2. Biochemical Tests:

Biochemical Tests test were carried out for the identification of the pathogen and the results are given in the table 1.

Ten isolates were selected from each plant for the characterization of biochemical properties out of which all the isolates shows positive results in utilization of carbohydrate, Arginine hydrolysis, starch hydrolysis, Tween 80 and Tyrosinase activity and all the isolates were negative for Aesculin hydrolysis and Kovac's oxidase. Some variations were recorded in Levan production and gelatin liquification. Strains RSC5 and RSC6 shows weakly positive in Levan and isolates RSC4 (Bho), RSC9 (Nan2), RSP4 (Bho), RSP9 (Nan2) showed positive result in gelatin liquefaction.

3.3 Biovar determination tests: On the basis of utilization of the Hexose sugar and Hexose alcohols, hypersensitivity reaction the isolates are classified into different races and biovars.

On the basis of the utilization of the alcohols and hypersensitivity reaction all the isolates produced the symptoms on the leaves of *Nicotiona tobaccum* after 48 hrs incubation at 30^0C. ten of the isolates from chilli belonged to race 1 and biovar 3 were as the other ten from potato belonged to race 3 and biovar 2 (Table 2).

3.4 Rep-PCR Profile: Rep PCR fingerprint profiles of 20 isolates of *R. solanacearum* from Marathwada region was analyzed by primers show repetitive patterns of bands. The DNA fingerprinting pattern of the 20 strains shown in the figure, the pattern corresponding to the conserved sequence of Repetitive element was obtained. The REP-PCR fingerprinting were resolved on agarose gel and showed total 17 bands in the gel. The banding profiles showed the bands in the range of 100bp-3000bp. The intensity of bands varied. Among the 17 bands, were 9 bands were common in all strains with molecular weight 300bp, 700bp, 900bp, 1300bp, 1600bp, 2000bp, 2300bp, 2500bp and 3000bp while remaining 8 bands are distinct. Five bands with high intensity were in the range of 900bp to 3000bp.

3.5 Cluster analysis :

The dendrogram generated is shown in figure 2. The first cluster had two sub clusters. The first sub cluster consisted of strains RSC2, RSC1, RSC3, RSC9, and RSC5 which showed maximum genetic similarity. Second sub-cluster consisted of RSC10 and RSC4 and RSC7, RSC6, and

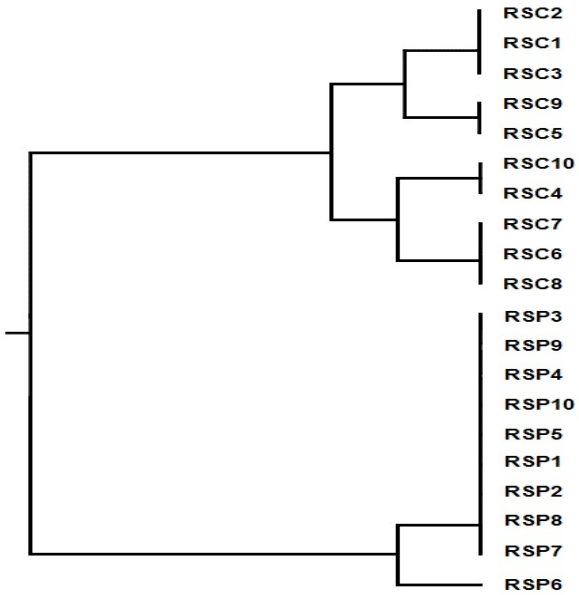

Figure 2: Dendrogram based on the rep-PCR of *Ralstonia solanacearum* by REP primer

RSC8 showed the maximum genetic similarity. Second cluster had isolates of host plant potato. Nine isolates of potato RSP3 RSP9, RSP4, RSP10, RSP5, RSP1, RSP2, RSP8, and RSP7 strains showed maximum genetic similarity.

[IV] DISCUSSION

The present work shows that 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 Marathwada, Maharashtra morphologically bearing resemblance to those isolated from other regions of the world [13] by producing typical white coloured fluidal colonies with pink centres and irregular in shape[7]. *R. solanacearum* is reported as one of the most destructive plant pathogen among bacterial plant pathogens [6]. The isolated bacteria are gram negative rods, the most reliable appearance of the pink centered colonies on TTC medium [5] however the biochemical characteristics and pathogenicity test confirmed the identity of the *R.*

solanacearum strain. Although the disease usually progresses until complete wilting and collapse of the plant, expression of the symptoms and rate of disease development may vary depending on host susceptibility and the aggressiveness of the pathogenic strain. In the cases where an infected plant does not show wilting, characteristic external symptoms may be dwarfing and stunting of the plant. The reliable Hypersensitive test was carried on *Nicotiona tobaccum* (Tobacco plant) and all strains induced a typical hypersensitive reaction (HR) in tobacco leaves and pathogenicity test were used for detection of the virulence of the isolates. Most of the strains were highly virulent and moderately virulent few of strain were less virulent. Diverse virulence of bacterial blight pathogen has been reported from different parts of the world. Earlier studies from the region of Marathwada showed that the strains mostly are the virulent [14]. Although in this study, races and biovar determination tests were also carried out as described by Haywards [7] and with the support of the study it is determined that the bacterial strain infects different plants, belongs to different Races and Biovars. The present study observed that the isolates of chilli plants belongs to race 1 and biovar 3 and the bacteria isolated from potatoes belongs to race 3 and biovar 2.

DAN fingerprinting was used to assess the diversity and genetic relationships among *R. solanacearum* isolates using Rep-PCR fingerprinting [15]. The characterized isolates by Rep-PCR technique using rep primer sets has provided enhanced capability to characterize and classify strains and assessment of the genetic diversity of populations was easily carried out. The diversity of large populations can be assessed in a relatively efficient manner using rep-PCR based genomic fingerprinting methods. The bacterial wilt cause yellowing of leaves and the ooze in the vascular tissue is observed in case of the leaves in developed stage sometimes necrosis reports and in serious condition results in sudden death of the plant whereas in case of the tuber like Potato bacterial ooze excretion from the freshly cut tuber is reported [16]. In severe condition, the black heart disease of potato also observed.

This variation in genetic diversity in natural population of this *R. solanacearum* could be a result of combination of many processes that may include genetic recombination, gene inactivation and mutation. Marathwada region of Maharashtra being a very small region such factors may be responsible for *R. solanacearum* diversity. The isolates associated with chilli belonged to race 1 and biovar 3 whereas, race 3 and biovar 2 was associated with potato indicating prevalence of genetic diversity among the isolates.

[V] CONCLUSION

R. solanacearum from Marathwada region of Maharashtra affect many vegetables grown in the region. *R. solanacearum* diversity prevails in the region as evident from morphologyical and biochemical test. Rep-PCR analysis showed the diversity within the strains. The isolates belonged to race 1 and biovar 3 in chilli and race 3 and biovar 2 in potato.

ACKNOWLEDGEMENT

Asrar Siraj thanks University Grant Commission New Delhi, for providing Maulana Azad National fellowship, also the Director of School of Life Sciences for providing necessary requirements at the school and Principal, Yeshwant Mahavidyalaya Nanded for their help.

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Isolates	Kovac's Oxidase	Utilization of carbohydrate	Levan production	Arginine hydrolysis	Aesculin hydrolysis	Starch hydrolysis	Tween 80	Tyrosinase activity	Gelatin liquification
RSC1(Loh)	-	+	+	+	-	+	+	+	+
RSC2(Kan)	-	+	+	+	-	+	+	+	+
RSC3(Nan)	-	+	+	+	-	+	+	+	+
RSC4(Bho)	-	+	+	+	-	+	+	+	++
RSC5(Ard)	-	+	±	+	-	+	+	+	+
RSC6(Bil)	-	+	±	+	-	+	+	+	+
RSC7(Nae)	-	+	+	+	-	+	+	+	+
RSC8(Nar)	-	+	+	+	-	+	+	+	+
RSC9(Nan2)	-	+	+	+	-	+	+	+	++
RSC10(Had)	-	+	+	+	-	+	+	+	+
RSP1(Loh)	-	+	+	+	-	+	+	+	+
RSP2(Kan)	-	-	+	+	-	+	±	+	+
RSP3(Nan)	-	+	+	+	-	+	+	+	+
RSP4(Bho)	-	-	+	+	-	+	+	+	++
RSP5(Ard)	-	+	+	+	-	+	+	+	+
RSP6(Bil)	-	+	+	+	-	+	+	+	+

RSP7(Nae)	-	+	+	+	-	+	+	+	+
RSP8(Nar)	-	+	+	+	-	+	+	+	+
RSP9(Nan2)	-	-	+	+	-	+	+	+	++
RSP10(Had)	-	+	+	+	-	+	±	+	+

"-" = negative, "+" = positive, "++" = strongly positive, "±" = weakly positive

Table 1: Results of Biochemical test of the strains:

Isolates	Dextrose	lactose	Maltose	Mannitol	Sorbitol	Dulcitol	Race	Biovar
RSC1(Loh)	+	+	-	+	+	+	1	3
RSC2(Kan)	+	+	-	+	+	-	1	3
RSC3(Nan)	+	+	-	+	+	-	1	3
RSC4(Bho)	+	+	-	+	+	-	1	3
RSC5(Ard)	+	+	-	+	+	-	1	3
RSC6(Bil)	+	+	+	+	+	-	1	3
RSC7(Nae)	+	+	+	+	+	-	1	3
RSC8(Nar)	+	+	+	+	+	-	1	3
RSC9(Nan2)	+	+	-	+	+	+	1	3
RSC10(Had)	+	-	-	+	+	-	1	3
RSP1(Loh)	+	+	+	+	+	+	3	2
RSP2(Kan)	+	+	+	+	+	+	3	2
RSP3(Nan)	+	+	+	+	+	+	3	2
RSP4(Bho)	+	+	+	+	+	+	3	2
RSP5(Ard)	+	+	+	+	+	+	3	2
RSP6(Bil)	+	+	+	+	+	+	3	2
RSP7(Nae)	+	+	+	+	+	+	3	2
RSP8(Nar)	+	+	+	+	+	+	3	2
RSP9(Nan2)	+	+	+	+	+	+	3	2
RSP10(Had)	+	+	+	+	+	+	3	2

"-" = Negative, "+"= Positive

Table: 2 Utilization of Hexose sugar and Hexose alcohol by chilli / Potato

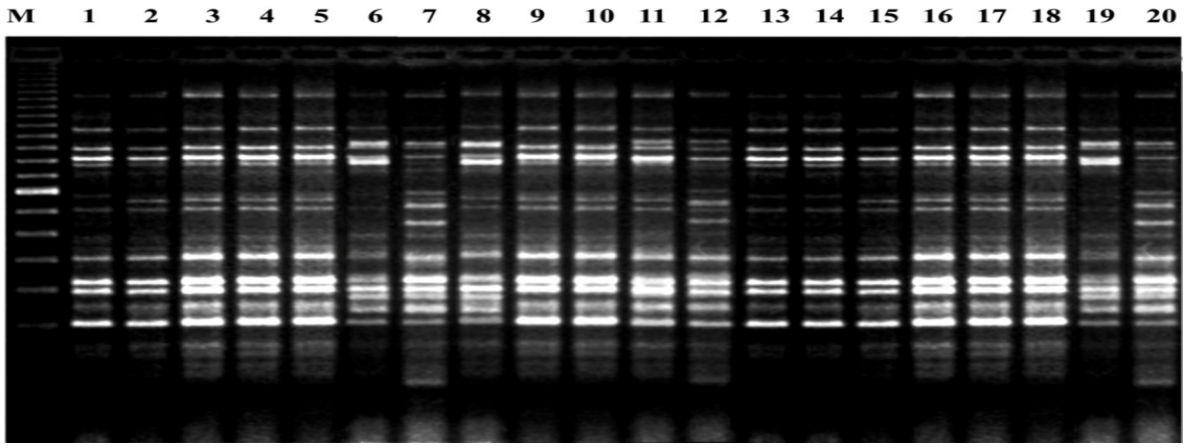

Figure 1: Repetitive DNA fingerprinting of *Ralstonia solancearum* isolates using REP primers(1-RSC2(Khandar), 2-RSC1(Loha), 3-RSC3(Nanded), 4-RSC9(Nanded), 5-RSC5(Ardhapur), 6-RSC10(Hadgaon), 7-RSC4(Bhokar), 8-RSC7(Naegaon), 9-RSC6(Biloli), 10-RSC8(Narsi), 11-RSP3(Nanded), 12-RSP9(Nanded), 13-RSP4 (Bhokar), 14-RSP10 (Hadgaon), 15-RSP5(Ardhapur), 16-RSP1(Loha), 17-RSP2(Khandar), 18-RSP8(Narsi), 19-RSP7(Naegaon), 20-RSP6(Biloli).