Journal of Tropical Agriculture 41 (2003): 33-37

DETECTION OF *RALSTONIA SOLANACEARUM* RACE 3 CAUSING BACTERIAL WILT OF SOLANACEOUS VEGETABLES IN KERALA, USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

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Abstract: Nine strains of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* isolated from bacterial wilt affected plants of brinjal, chilli and tomato in three different agroclimatic zones of Kerala were compared based on the utilization of carbohydrates, hypersensitivity reaction on capsicum leaves and RAPD analysis. Among these, six isolates were grouped into Biovar III and three, into Biovar IIIA. The isolates belonged to Races 1 and 3. RAPD analysis with 10 decamer primers revealed a high degree of polymorphism among the isolates. The primer OPF 8 yielded a unique band of 1.45 kb size for Race 3. This could be considered as a marker for rapid identification of Race 3 isolates of *R. solanacearum*.

Key words: Bacterial wilt, biovar, race, *Ralstonia solanacearum*, RAPD, solanaceous vegetables

INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* is one of the most serious diseases of crops in tropics, subtropics and warm temperate regions of the world. In Kerala, the disease is widespread in solanaceous vegetables, cucurbits and ginger. The pathogen exhibits wide variability and diversity which often confuses plant breeders by breaking down of resistance of varieties evolved through extensive breeding programmes. Isolates of *R. solanacearum* are generally grouped, based on utilization of disaccharides and hexose alcohols, into biovars and based on host range and hypersensitivity reaction on specific hosts, into races. However, the recent trend in pathotyping is by molecular techniques like polymerase chain reaction (PCR), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) (Fegan *et al.*, 1997; Raymundo *et al.*, 1997 and Thwaites *et al.*, 1999). In this paper, an attempt has been made to use RAPD as a tool to characterize *R. solanacearum* isolates obtained from Vellanikkara (Thrissur), Kumarakom (Kottayam) and Ambalavayal (Wayanad), representing three agroclimatic zones of Kerala. The objective was to develop a method of identification of strains that could quickly and clearly distinguish between biovars / races cultured under *in vitro* conditions which could later be applied to the study of indigenous populations in soil / bacterial ooze from the infected plant.

MATERIALS AND METHODS

Brinjal, chilli and tomato plants showing typical bacterial wilt symptoms were collected from the three locations and the pathogen was isolated on tetrazolium chloride (TZC) medium (Kelman, 1954). Typical mucoid, creamy white colonies with pink centre after 48 h of incubation were transferred into sterile water and maintained at 28-30°C. Pure cultures of the isolates were subjected to various biochemical tests.

The isolates were differentiated into biovars, based on their ability to utilize disaccharides (lactose, maltose and cellobiose) and hexose alcohols (mannitol, sorbitol and dulcitol), according to Hayward (1964), He *et al*. (1983) and Kumar *et al*. (1993).

Race differentiation was done based on host range and hypersensitivity reaction (HR) on capsicum by leaf infiltration technique used by Lozano and Sequeira (1970). The bacterial suspensions (OD 0.3 at 600 nm) were infiltrated into the intercostal region on the undersurface of capsicum leaves using a disposable syringe with its needle removed. The barrel of the syringe was placed in close apposition to the undersurface of the leaf and the pressure was gently applied. The plants were then examined at 14, 24, 48, 72 h, up to 10 days.

For RAPD analysis, the genomic DNA was isolated from the nine isolates following Girija (1999). Quality of DNA was assessed by agarose gel electophoresis on 1% agarose in Hoefer electrophoresis system and quantity of DNA calculated from optical density in a Spectronic 20 Genesys 5 spectrophotometer based on the calculation:

1.0 OD at 260 nm = 50 μg of DNA / ml of the sample

Ten decamer primers under OPE, OPP, OPF and OPAH series procured from Operon Technologies, Inc., USA, were tested for DNA amplification by RAPD, for producing polymorphism among the strains. Each reaction was set up in 25 µl mixture consisting of 2.5µl 1x PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.6 u Taq DNA polymerase, 10 pM primer and 50 ng of template DNA. This mix was overlaid with 25 µl mineral oil. DNA amplification consisted of 45 cycles of denaturation at 94°C for 1 min, annealing at 37.5°C for 1 min and extension at 72°C for 2 min in a peltier thermal cycler (MJ Research, USA). The amplified products were separated on 1.5% agarose gel in 1x TAE buffer at 120V and visualized on a UV transilluminator (Hero Lab, USA).

RESULTS AND DISCUSSION

Isolation of the pathogen on TZC medium yielded creamy white mucoid colonies with pink centre. This medium can distinguish virulent strains from the mutant avirulent ones since the latter produce non-mucoid, butyrous colonies. Based on cultural, morphological and biochemical characters, the pathogen was identified as *Ralstonia solanacearum*.

Six among the nine isolates, which utilized all the carbohydrates were grouped into Biovar III and rest three, that did not utilize dulcitol, were designated as Biovar IIIA. In the hypersensitivity reaction test, six isolates produced no visible symptoms on inoculated capsicum leaves 24 h

Table 1. Differentiation of *Ralstonia solanacearum* isolates into biovars and races

Isolate	Utilization of carbohydrates						Biovar	Race
	Lactose	Maltose	Cellobiose	Mannitol	Sorbitol	Dulcitol		
V1	+	+	+	+	+	+	III	1
V2	+	+	+	+	+	+	III	1
V3	+	+	+	+	+	-	IIIA	1
K1	+	+	+	+	+	+	III	1
K2	+	+	+	+	+	-	IIIA	1
K3	+	+	+	+	+	+	III	1
A1	+	+	+	+	+	+	III	3
A2	+	+	+	+	+	-	IIIA	3
A3	+	+	+	+	+	+	III	3

Location: V = Vellanikkara, K = Kumarakom, A = Ambalavayal; Host: 1 = Brinjal, 2 = Chilli, 3 = Tomato

Table 2. Quality and quantity of genomic DNA as revealed by spectrophotometry

Isolate	Optical density		OD_{260}/OD_{280}	Quality of DNA	Quantity of DNA (µg/ml)
	260 nm	280 nm			
V1	0.134	0.074	1.81	Good	3.70
V2	0.085	0.050	1.70	Fairly good	2.50
V3	0.150	0.081	1.85	Good	7.50
K1	0.159	0.088	1.80	Good	7.95
K2	0.133	0.074	1.80	Good	6.65
K3	0.052	0.028	1.85	Good	2.60
A1	0.100	0.056	1.78	Good	5.00
A2	0.080	0.042	1.90	Fairly good	4.00
A3	0.064	0.036	1.78	Good	3.20

Location: V = Vellanikkara , K = Kumarakom, A = Ambalavayal; Host: 1 = Brinjal, 2 = Chilli, 3 = Tomato

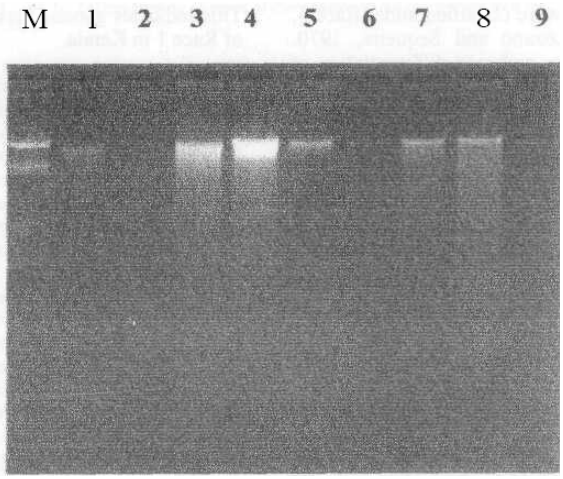

Fig1. Genomic DNA from *Ralstonia solanacearum* isolates on agarose gel

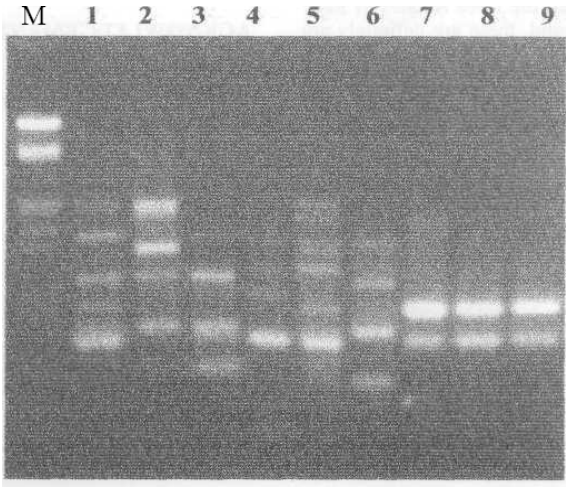

Fig 2. RAPD profile of *R. solanacearum* isolates with OPF 8
[M = Molecular weight marker, Lane 1 = V1, Lane 2 = V2, Lane 3 = V3, Lane 4 = K1, Lane 5 = K2, Lane 6 = K3, Lane 7 = A1, Lane 8 = A2, Lane 9 = A3]

after inoculation, but a dark brown necrotic lesion surrounded by a yellow halo appeared after 36 h in the infiltrated area. This was followed by extensive wilting and yellowing of leaves. These were classified as Race 1. Three isolates caused only a yellow discolouration by 48 h af-

ter inoculation and were classified under Race 3, as suggested by Lozano and Sequeira, 1970. The results of biovar and race differentiation of the isolates are presented in Table 1.

Genomic DNA extaction procedure yielded good quality and quantity of DNA as revealed by spectrophotometry and agarose gel electophoresis (Table 2 and Fig 1). In RAPD, all the 10 primers tested exhibited high polymorphism with respect to different isolates. The number of bands ranged from zero to 10 and size of amplified fragments, from 0.1 to 5 kb. DNA amplification with different primers revealed great diversity in the population of *R. solanacearum* Biovars III and IIIA. None of the primers yielded bands specific to any of the two biovars. There are reports on the genetic diversity among the biovars of the pathogen (Jaunet and Wang, 1997). However, OPF 8 was found to yield a unique band of size 1.45 kb for isolates A1, A2 and A3, which belonged to Race 3. This band was absent in all other isolates (Fig. 2). Hence this could be considered as a molecular marker for identification of Race 3 of *R. solanacearum* in Kerala. Though all the three isolates of Race 3 share a common band, these are definitely not the same strain as these are not cross-inoculable and exhibited different banding pattern with other primers (data not given). No such unique band could be observed in the case of Race 1. This indicates genetic variability among isolates of Race 1 in Kerala.

Molecular detection methods are considered to be highly specific, sensitive and rapid. DNA amplification can detect even minute genetic differences among closely related strains, which may otherwise resemble one another in cultural, morphological and biochemical characteristics. Use of random or arbitrary primers for identification of individuals in differing populations is based on the theory that if any oligonucleotide is chosen at random, its distribution within the genome of differing individuals will vary. These techniques have been used for pathotyping of *R. solanacearum* even without isolating the pathogen from infected plant. Bacterial DNA extracted directly from soil could be used for amplification by PCR in pathotyping (Ito *et al.,* 1997). Such rapid procedures could replace the long and tedious process of culturing the bacteria and conducting laborious microbiological tests. However, more isolates are being screened for recommending it as a molecular marker for Race 3.

ACKNOWLEDGEMENT

This paper forms a part of M.Sc. (Ag.) thesis of the first author submitted to the Kerala Agricultural University.

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