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Genetic Diversity and Pathogenicity of *Ralstonia solanacearum* E.F. Smith in Tomato (*Lycopersicon esculentum* Mill.) in Kandy, Matale, and Monaragala Districts

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ABSTRACT. *Bacterial wilt, caused by Ralstonia solanacearum E.F. Smith, is one of the most destructive diseases of tomato (Lycopersicon esculentum Mill) in Sri Lanka. Twenty isolates of R. solanacearum from tomato plants grown in eleven different agro climatic zones from selected districts (Kandy, Matale, Monaragala) were characterized in terms of pathogenicity on susceptible check variety Marglobe and in molecular level using random amplified polymorphic DNA (RAPD) method. Each isolate was inoculated to ten Marglobe plants using root inoculation method. Plants were inoculated with 50 ml of 48 hours old cultures with 10^8 CFU by pouring into the root zone. Data collection was done at 3 days intervals up to 30 days. Sixteen isolates were highly virulent and four isolates were moderately virulent on Marglobe under green house conditions. Isolate collected from Hanguranketa (Maoya) area showed the highest relative pathogenicity value (5) where the lowest value was recorded by the isolate collected from Randenigala area (3.8). It is suggested that the isolate collected from Hanguranketa could be used for screening of existing tomato germplasm in Sri Lanka for bacterial wilt resistance. Genetic diversity of isolates was assessed using RAPD method with ten selected decamer Operon primers, namely OPA-02, OPA-03, OPA-04, OPA-14, OPC–15, OPD-07, OPD-08, OPD-10, OPD–15, and OPF-12. A higher degree of polymorphism was observed even in the isolates collected from the same agro-ecological zone. RAPD data did not reveal clear relationship between genetic differences and the virulence of the pathogen, but indicated that a higher genetic diversity of the pathogen existed in the three districts considered.*

INTRODUCTION

The cultivated area under major vegetables in Sri Lanka increased from 71869 ha to 74151 ha during the period from 1997 to 1999. Over the same period, the total area under the tomato cultivation increased from 5079 ha to 5788 ha (Anon, 1997, 1999). The area under tomato cultivation is expected to increase

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further in the future and one of the major obstacles in increasing tomato cultivation is bacterial wilt.

Bacterial wilt is caused by a soil borne pathogen *Ralstonia solanacearum* E.F. Smith. This disease causes heavy losses in the mid and low country wet and intermediate zones in Sri Lanka (Kelaniyangoda, 1995). Farmers in these areas experienced considerable economic losses when susceptible crop cultivars are grown.

R.alstonia solanacearum can spread within and between countries by soil, by water, and by latently infected planting material (Hayward, 1991). Therefore, rapid and highly sensitive detection methods of *R. solanacearum* are required for quarantine to reduce field losses and limit the spread of bacterial wilt (Seal, 1995). Traditional methods for detecting *R. solanacearum* depend on a series of biochemical tests on purified colonies. This identification procedure is time consuming for quarantine and other diagnostic laboratory purposes. Nucleic acid probes and polymerase chain reaction (PCR), on the other hand, have been widely reported as tests that can be performed without culturing. Diagnostic tests based on PCR amplification of plant pathogens from environmental samples have shown that rapid and specific detection of very low numbers of the pathogen can be achieved (Seal, 1995; Williams *et al.*, 1990; Gillings, 1992).

Ralstonia solanacearum is a complex and heterogeneous species. Disease control is difficult because of broad host range, wide spread distribution and vast genetic diversity of *R. solanacearum* (Hayward, 1991). The variability in its biochemical properties, serological reactions, membrane proteins and phage susceptibility, show that the species is composed of a number of quite distinct strains. The effect of location as well as the location and variety interaction on virulence has been shown to be highly significant (Hanson and Wang 1996; Elphistone, 1994; Narayan and Peter 1996; Prior *et al.*, 1990). Such diversity of pathogen and location specific nature of resistance poses a number of problems, particularly in the production of resistant cultivars (Acosta *et al.*, 1995), consequently resistant cultivars produced in one geographic area may not be resistant to bacterial strains in another area. The bacterial wilt resistant tomato varieties (KWR, T245, T146) released by the Department of Agriculture were popular among farmers in the past; however, some of these varieties are now susceptible to bacterial wilt in some areas.

Strains isolated from different locations are not uniform and differ from each other in many characteristics (Kelman, 1954). Therefore, it is necessary to identify a more virulent strain for screening purposes of available tomato germplasm in Sri Lanka. For effective quarantine and detection measures against *R. solanacearum*, it is necessary to identify genetic diversity of the pathogen in Sri Lanka.

MATERIALS AND METHODS

Location

The research was conducted at the Plant Genetic Resources Center, Gannoruwa, Peradeniya from January to September 2003.

Collection of pathogen

Surveying and sampling were carried out at 20 locations in 11 different agro ecological regions in Kandy, Matale and Monaragala districts during February to May 2003 (Table 1). The tomato (*Lycopersicon esculentum* Mill.) plant samples, which showed typical symptoms of bacterial wilt, were collected from the farmer's fields of randomly selected locations. Two plants were sampled from each location and the disease severity was recorded. Infected plant stems were thoroughly washed and placed in test tubes (one plant each) containing sterilized distilled water. The bacterial ooze from these tissues was allowed to stream into sterilized water. A loop full of this milky bacterial suspension was streaked on culture plates containing 10ml of SPA (K_2HPO_4 0.5 g; $MgSO_4.7H_2O$ 0.25 g; Peptone 5.0 g; Sucrose 20.0 g; Agar 20.0 g; and distilled water 1L) medium and pure cultures were obtained after 2-3 subculturing steps.

Table 1. Samples collected from different locations in Sri Lanka.

Sample	Location	Sample	Location
01	Mathale–Walawela-WM3b	11	Kandy – Marassana-IU2
02	Matale-Unaveruwa-WM3b	12	Kandy – Marassana-IU2
03	Matale-yatawatthe-WM3b	13	Kandy – Madadeniya-IM3c
04	Matale-Naula- IM3b	14	Kandy – Gannourwa-WM2b
05	Matale –Naula-IL3a	15	Kandy – Gannoruwa-WM2b
06	Matale-Bomballa-IL3a	16	Kandy – Randenigala-IM1b
07	Matale- Dambulla-DL1c	17	Kandy – Randenigala-IM1b
08	Kanday-Agalpala-IM3c	18	Monaragala – Nakkala-IL1b
09	Kandy – Maoya-IM3c	19	Monaragala-Badalkumbura-IL1c
10	Kandy– Neelawela-IM1c	20	Monaragala –Hindagoda-IL1b

Virulency test

Virulence test was conducted for isolated cultures from 20 different locations. Kelmans TZC medium (Kelman, 1954) (1000 ml distilled water; 10 g dextrose; 10 g peptone; 1g casimic acid; 18 g agar) was used to identify virulent cultures. Before pouring the warm liquid medium into petridishes, 1% sterile 2,2,3-triphenyl-tetrazolium chloride (TTC) was added as 5 ml per 1000 ml.

A diluted bacterial suspension was streaked on the surface of TTC agar medium with a wire loop. After 36 hours of incubation at 32°C, colour of the colonies was examined in the day light.

Pathogenicity test

Inocula for pathogenicity tests were prepared with 48 hours old cultures grown on Kelmen medium without TZC grown at 28-30°C. Colonies were suspended in sterilized water to get $1x10^8$ CFU/ml using a spectrophotometer, transmission at A600=0.1 (Somodi *et al.*, 1992) and were used for inoculation immediately after preparation.

All the soil mixtures used in the research were steam sterilized for two hours. Ten plants of (fourteen days old) seedlings of Marglobe (Susceptible check variety) and KWR (Resistant check variety) were grown in the polythine bags (20 x 20cm) containing sterilised soil mixture as one plant per bag and maintained under green house conditions. Disease was not recorded for a period of one month. Therefore, Marglobe was selected for the study and plants were grown from seeds in trays containing sterilized soil mix consisting of organic matter: soil (2:1). Fourteen days later vigorous seedlings were transferred (one plant per bag) to polythine bags (20 cm x 20 cm) consisting sterilized soil mixture (organic matter: soil 2:1). Plants were maintained at green house where temperature was at 26-30°C and the relative humidity ranged from 78-95%. Five weeks old seedlings were used for inoculation.

Root inoculation was done as described by Somodi *et al.*(1992). Root zone was damaged using a sterilized scalpel and 50 ml of prepared culture was introduced to each plant. Each strain was injected into 10 Marglobe plants. Plants were kept at the green house and all the agronomic practices were done according to the recommendations of the Department of Agriculture. Plants were evaluated at 3-day intervals for 30 days after inoculation.

Disease severity was recorded on a 1-5 scale: 1 = no visible symptoms; 2 = 1-25% of the plant is wilting; 3 = 26-50% wilt; 4 = 51-75% wilt and 5 = more than 75% wilt. Virulence of each strain was rated according to the mean performance of 10 plants from each host after 30 days of inoculation: virulence = 1.0; low virulence = 1.1-2.5; medium virulence = 2.6-4.0 and high virulence = 4.1-5.0 (Kelman and Person, 1961).

Genetic diversity of isolated cultures using RAPD technique

Extraction of bacterial DNA

Overnight grown cultures of *R. solanacearum* in Liquid Broth were used for DNA extraction. About 1.5 ml of culture from each isolate was measured and centrifuged at 12000 rpm. Then the pellet was resuspended and lysed in 200 µl of lysis buffer (40 µM Tris-acetate at pH 7.8, 20 µM Sodium acetate, 1 µM EDTA, 1% SDS) by vigorous pipetting. Then, 66 µl of 5 M NaCl was added and mixed well and the viscous mixture was centrifuged at 12000 rpm for 15 min. The clear supernatant was transferred into a new vial. An equal volume of chloroform was added to the supernatant and tubes were gently inverted until a milky solution was completely formed. The solution was then centrifuged at 12000 rpm for 3 minutes and supernatant was transferred into a new vial. DNA was precipitated with 2

volumes of 100% ethanol at –20°C for 10 min. Then the pellet was washed with 70% ethanol and dried for 30-45 min. the DNA pellet was dissolved in 50 µl of distilled water and stored at –20°C.

Primers and PCR conditions

For RAPD analysis, PCR amplifications were carried out in 12.5 µl volumes (Williams *et al.,1990*), which contained 1.25 µl of 10xPCR buffer, 2 µl dNTP mixture (2.5 mM), 2 µl of primer (33mg/ml) and 2 µl of template DNA (10 ng). Amplification was performed in air thermal cycler programmed for one cycle of 3 min at 94°C, 40 cycles of 1 min at 93°C, 3 min at 35°C, 2 min at 72°C, one cycle of 10 min at 72°C. Ten different Operon primers of ten bases in length were used (Table 2). Amplified DNAs were loaded into 1.4% agarose gels and electrophorased in 0.5 TBE buffer at 5v/cm for 80-90 minutes. Gels were stained with ethidium bromide and photographed under UV light.

Data analysis

The RAPD banding pattern for each primer were of scored by visual observation. The presence of amplified product (band) in each position was recorded (1-presence and 0-absence).

Cluster analysis was performed to obtain a tree diagram (dendogram) by the SAS computer package (Version 08, procedure PROC CLUSTER, method = average pseudo, the PROC TREE procedure).

Table 2. Primers used for RAPD analysis.

Primer	Sequence (5' to 3')	Primer	Sequence (5' to 3')
OPA-02	TGCCGAGCTG	OPD-07	TTGGCACGGG
OPA-03	AGTCAGCCAC	OPD-08	GTGTGCCCCA
OPA-04	AATCGGGCTG	OPD-10	GGTCTACACC
OPA-14	TCTGTGCTGG	OPD-15	CATCCGTGCT
OPC-15	GACGGATCAG	OPF-12	ACGGTACCAG

RESULTS AND DISCUSSION

Virulency test

The virulent colonies were of irregular shape, slimy, white with pale pink centers. The avirulent colonies were small and round, cream and deep red with a thin lighter border. Virulent colonies were selected and suspension of the cultures was prepared in sterile distilled water and stored at -21°C for pathogenicity studies and molecular studies.

Pathogenicity test

Under the green house conditions, sixteen of twenty isolated cultures were highly virulent for the susceptible test cultivar Marglobe. However, cultures isolated from some areas of Matale (Walawela, Unawerua and Yatawatte) and Randenigala showed medium virulence, and the disease was not severe even in the farmer's fields in those locations. The highest relative pathogenicity was obtained in the culture isolated from Hanguranketha area (Maoya) with score value 5.0, where high disease severity was observed even in the field (Table 3).

Table 3. Relative pathogenicity of collected isolate.

Isolate	Score	Relative pathogenicity	Isolate	Score	Relative pathogenicity
1	4.0	Medium	11	4.3	High
2	3.9	Medium	12	4.5	High
3	4.2	High	13	4.8	High
4	4.7	High	14	4.6	High
5	4.8	High	15	4.7	High
6	4.5	High	16	3.6	Medium
7	4.6	High	17	3.8	Medium
8	4.9	High	18	4.5	High
9	5.0	High	19	4.6	High
10	4.9	High	20	4.7	High

Relative pathogenicity (=sensitivity) measured as Kelman and Person (1961).

RAPD analysis

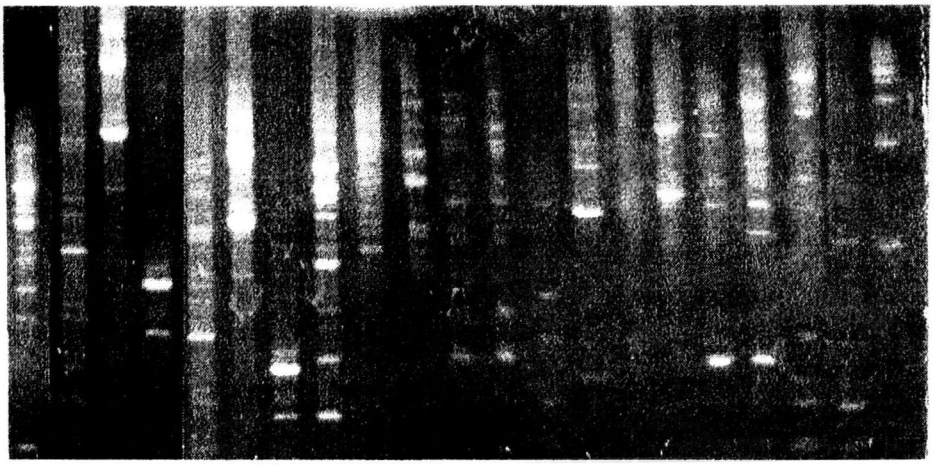
A total of 650 bands were recorded. According to Sambrook *et al.* (1989) the fragment size to be distinguished in 1.4 % agarose gels ranged approximately from 0.2 – 0.3 kb. Since the thick fragments may be a combination of several bands, extremely thick bands were ignored in scoring. Therefore, finally 524 bands obtained from 20 isolates using ten primers were used and all bands showed polymorphism (Fig. 1). This is similar to the results obtained by Cook and Sequriea (1991) which has indicated that different strains of *R. solanacearum* often contain DNA that is not present in all members of the same race. All the primers produced relatively higher number of bands (Table 4).

Table 4. Data obtained in RAPD analysis.

Primer	Number of polymorphic bands	Fragment size (kb)
OPA-02	68	2-15
OPA-03	66	3-10
OPA-04	69	1-12
OPA-14	62	1-13
OPC-15	32	2-14
OPD-07	65	3-15
OPD-08	47	2-14
OPD-10	22	1-14
OPD-15	33	3-15
OPF-12	60	1-15

Cluster analysis

It has produced three main clusters at the average distance of 9.50 (Fig. 2). There is no relationship between the geographical location and the clustering. Even the isolates collected from different fields of same agro ecological zone have been clustered separately; i.e. isolates collected from Gannoruwa (14, 15) and from Randenigala (16, 17). There is no clear relationship between relative pathogenicity value observed and clustering.



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Fig. 1. RAPDs with OPF-12 Primer

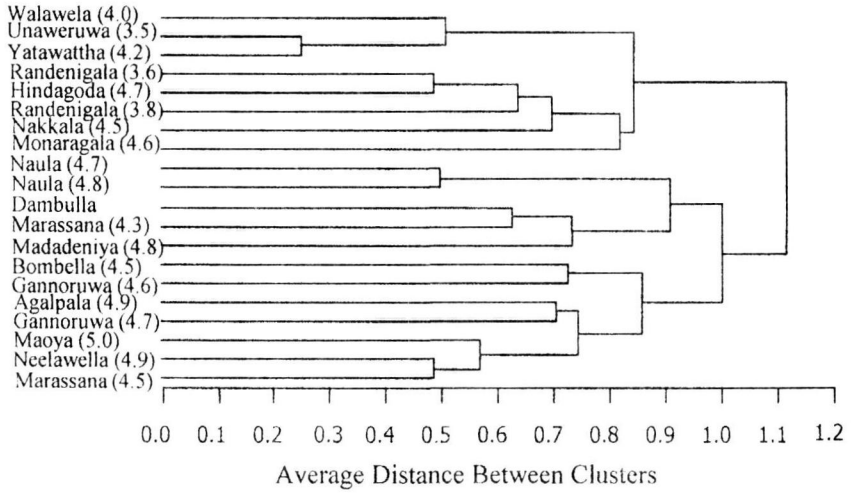


Fig. 2. Cluster diagram for 20 isolates classified by banding pattern of RAPD primers.

CONCLUSIONS

Sixteen of the collected isolates are highly virulent and four are moderately virulent on susceptible check variety Marglobe. It is suggested that the isolate collected from Hanguranketa (Maoya) can be used for screening of existing tomato germplasm in Sri Lanka for bacterial wilt resistance since it gives the highest relative pathogenicity score value.

Genetic diversity of the pathogen is very high in Kandy, Matale and Monaragala districts. It is difficult to identify a common band for the collected isolates of *Ralstonia solanacearum* using RAPD technique with the Operon primers used in the study.

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