Typing of *Ralstonia solanacearum* isolated from tomato by antibiotic susceptibility, plasmid profiling and PCR-based techniques of RAPD and ERIC

(Pentaipan *Ralstonia solanacearum* yang diasingkan daripada tomato menggunakan kaedah pengaruhan antibiotik, profail plasmid dan teknik PCR seperti RAPD dan ERIC)

A.M. Sahilah*, S. Tosiah*, R. Son**, W.L. Ooi**, C.Y.K. Queen**, L.S.H. Jeffrey*, S.C. Chua** and M.T. Mohd. Senawi*

Key words: *Ralstonia solanacearum,* antibiotic susceptibility, plasmid profiles, RAPD-PCR and ERIC-PCR

Abstract
The epidemiological characteristics of *Ralstonia solanacearum* isolated from tomato plants in Sarawak and Selangor were studied. The epidemiological analysis of the strains was carried out through antibiotic resistant pattern, plasmid profiles, randomly amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC) methods. Six strains were susceptible to all antibiotics tested, whereas the 10 strains that were resistant to one or more antibiotics were grouped into six antibiotic resistance patterns. Small single plasmid of 7.2 Mda and 9.2 Mda were detected among the nine plasmid containing strains, enabling them to be grouped into only two plasmid patterns.

In the polymerase chain reaction, based methods using RAPD and ERIC, two strains were untypable by RAPD, whereas all were typable by ERIC. In this study, a wide diversity of *R. solanacearum* strains was examined. ERIC analysis demonstrated the clonal relationship between isolates from tomato plants in Sarawak and Selangor. The existence of similar *R. solanacearum* strains in tomato plants from two very distant locations should be considered if tomato strain fingerprint results were to be used to help trace the vehicles for transmission.

*Strategic Resources Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301,
  50774 Kuala Lumpur, Malaysia
**Department of Biotechnology, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia,
  43400 UPM Serdang, Selangor, Malaysia
Authors' full names: Sahilah Abd. Mutalib, Tosiah Sadi, Son R., Ooi, W.L., Queen C.Y.K., Jeffrey, L.S.H.,
Chua S.C. and Mohd. Senawi Mohd. Tamin
E-mail: sahilah@mardi.my

Introduction

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most important plant diseases particularly to groundnut, ginger, banana and solanaceous crops such as potato, pepper and tomato. There are widespread bacterial diseases of plant in the tropics, subtropics and warm temperate regions of the world (Hayward 1991).

The typing of bacterial wilt associated with the exposure to *R. solanacearum* is of considerable importance in the surveillance of possible plant health risk of *R. solanacearum* and as a research tool for predictive value in epidemiological control and in plant quarantine. It also allows the identification of the presence of virulent strains, appearance of new strains and changing pattern of this plant disease to be monitored. This may be achieved by using highly discriminatory molecular technique methodologies, which allow rapid and sensitive differentiation between strains within a single species.

Various genotypic techniques have been developed. These include enterobacterial repetitive intergenic consensus (ERIC), random amplified polymorphic DNA (RAPD), amplified restriction fragment length polymorphism (AFLP), plasmid profiling and pulsed field gel electrophoresis. These approaches are very helpful and have been applied by many researchers to members of diverse bacterial genera, including *Rhizobium, Frankia, Staphylococcus, Legionella, Xanthomonas* and *Pseudomonas* (Gillings and Holley 1997) to investigate the genetic diversity, evolutionary and epidemiological relationships of the bacteria. Thus, attempt has been made to investigate the genetic diversity of plant pathogen bacteria of *R. solanacearum* using four techniques: antibiotic susceptibility, plasmid profiling, RAPD and ERIC.

Materials and methods
Bacterial strains

The plant isolates of *R. solanacearum* used in this study were isolated from tomato obtained from different fields in Jalan Kebun and Sri Serdang, Selangor and Jalan Sekama, Kuching, Sikong and Ta'ee in Sarawak, Malaysia. The samples were collected in July 1995. The stem of tomato plants exhibiting symptoms of typical wilt diseases were cut and washed with 10% (v/v) clorox and rinsed twice with sterile distilled water. The outer skin was cut. The inner flesh was ground and inoculated onto tetrazolium chloride (TZC) medium (Glucos 10 g/litre, peptone 10 g/litre, casein hydrolysate 1.0 g/litre, agar 15 g/litre and pH 6.8–7.0). The light pink colonies were picked-up and suspected colonies were identified using BIOLOG identification system (BIOLOG Inc.).

Bacterial growth and chromosomal DNA preparation

All strains were grown in Lauria-Bertani (LB) broth at 37 °C with shaking at 200 rpm overnight. Prior to amplification by RAPD and ERIC-PCR, total genomic DNA of the *R. solanacearum* strains were extracted using boil cell method. The cell suspension was placed into microcentrifuge tube and centrifuged at 10,000 rpm for 2 min. Supernatant was discarded. To obtain enough cell, the previous steps were repeated 3–4 times. The cell pellet was then washed twice with 1.0 ml of distilled water. The tubes were vortexed and centrifuged at 10,000 rpm for 5 min. The supernatant was discarded. Sterile distilled water (1 ml) was added and vortexed. The mixture was boiled for 5 min and immediately put in ice for 5 min. The suspension was centrifuged at 10,000 rpm for 5 min. A sample of 5 μl of supernatant was taken for ERIC and RAPD-PCR purposes (to be used immeadiately).

Plasmid isolation

All strains were grown in Lauria-Bertani (LB) broth at 37 °C with shaking at 200 rpm overnight. Small scale preparation of plasmid DNA from *R. solanacearum* strains was obtained using the rapid alkaline extraction procedure described by Sambrook et al. (1989). About 1.5 ml of culture broth was placed into microcentrifuge tubes and centrifuged at 10,000 rpm for 2 min. The supernatant was discarded and the pellet was resuspended thoroughly in 150 µl of GET [50 mM of glucose, 10 mM of ethylenediaminetetra-acetic acid (EDTA) and 25 mM of Tris-HCl; pH 8.0] buffer, followed by the addition of 175 µl of 2% (w/v) sodium dodecyl sulphate (SDS) and 175 µl of 0.4 M natrium hydroxide (NaOH).

The tube was inverted gently a few times and was left at room temperature for 5 min before 250 µl of cold 3 M potassium acetate (KAc) (29.44 g and 11.5 ml of acetic acid in 100 ml) was added. The tubes were then incubated at –20 °C for 10 min. After centrifugation for 5 min at 12,000 rpm to remove unwanted material, the supernatant was transferred to a new microcentrifuge tube. Ice cold propanol (750 µl) was added to aqueous phase and the mixture was spun down for 10 min at 12,000 rpm.

The supernatant was decanted and the pellet was washed with 500 µl of 70% ethanol by centrifugation for 5 min at 12,000 rpm at room temperature. The pellet was briefly air dried at room temperature, then dissolved in 40 µl of sterile distilled water and immediately used for electrophoresis. The approximate molecular mass of each plasmid was determined by comparing it to a plasmid of known molecular mass, *E. coli* V517 (Macrina et al. 1978).

Antibiotic susceptibility testing

Susceptibility to antimicrobial agents tested was carried out using the standard disc diffusion method (NCCLS 1997). Disc containing the following antibiotics was used: ampicillin at 10 mg, bacitracin at 10 µg, carbenicillin at 100 µg, cephalothin at 30 µg, nalidixic acid at 30 µg, kanamycin at 30 µg, rifampicin at 5 µg, streptomycin at 10 µg and tetracycline at 30 µg (BBL Sensi-Disc, Becton Dickinson). The plates were incubated for 24 h at 37 °C. The sensitivity or resistance of each isolate towards these antibiotics was determined by measuring the diameter of the inhibition zone around the antibiotic disc.

RAPD-PCR fingerprinting

A randomly designed 10-mer oligonucleotide set, designated as P1–P10 was obtained from the Genosys Biotechnologies Ins. (TX, USA). The P3 (5'-ACGGTGCCTG-3') and P5 (5'-GAGATCCGCG-3') were chosen for RAPD-PCR analysis because they yielded clear patterns. PCR assays were routinely performed in a 25 µl reaction mixture containing 20–30 ng of template DNA, 2.5 µl 10x PCR buffer, 1 unit *Taq* DNA polymerase, 0.2 mM primer, 2.5 mM $MgCl_2$ and 1 mM each of dCTP, dGTP, dATP and dTTP.

RAPD-PCR was carried out using a thermal cycler (Perkin Elmer 2400, Norwalk, USA). The cycling parameters were 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C for a total 45 cycles, with final cycle extending amplification conditions to 72 °C for 5 min. The PCR amplification products were visualised by running 10 µl of the reaction on 1.0% agarose gel in 1x TBE buffer (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA), detected by staining with ethidium bromide and photographed under UV transillumination. The 1 kb DNA ladder (Promega, USA) was used as a DNA size marker.

ERIC-PCR fingerprinting

The primers used were ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') as described by Gillings and Holley (1997). PCR amplification reactions consisted of

25 μl volume containing 10 ng of genomic DNA, 2.5 μl 10x PCR buffer, 2.5 unit *Taq* polymerase, 5 mM each of the forward and reverse primers, 2 mM MgCl$_2$, and 1 mM each of dCTP, dGTP, dATP and dTTP. Amplification was done using a Perkin Elmer 2400 thermocycler (Perkin-Elmer, Norwalk, USA) as follows: 95 °C for 5 min, 90 °C for 5 min, 55 °C for 1 min, 65 °C for 8 min and a final elongation step at 65 °C for 16 min at the end of 35 cycles. The amplification products were fractionated by electrophoresis using 1.2% agarose gel in 1x TBE buffer (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA), detected by staining with ethidium bromide and photographed under UV transillumination. The 1 kb DNA ladder (Promega, USA) was used as a DNA size marker.

Interpretation of RAPD and ERIC-PCR fingerprinting

Scanned images were analysed using the comparative analysis of electrophoresis pattern of GelCompar (Kortrijik, Belgium). Bands were assigned on a presence-absence basis, regardless of intensity, using the cursor to mark the location. The software estimated band sizes for all data sets. Pairwise similarity indices were estimated between isolates using a simple similarity index that in the number of bands shared by two fingerprints divided by the total number of unshared bands. Intraspecies of *R. solanacearum* isolates were clustered using average linkage (UPGMA, unweighted group pair method with arithmetic averages) and displayed in dendrogram form.

Results

Antibiotic susceptibility test

The 16 strains of *R. solanacearum* were tested for their susceptibility towards 14 antibiotics in *Table 1*. Antibiotic susceptibility analysis showed that 10 strains were resistant to one or more antibiotics (62.5%), producing six antibiotic resistant patterns. Whereas, six strains were susceptible towards all antibiotics tested

(37.5%). They were highly resistant to bacitracin (56.2%). Resistance towards rifampicin and penicillin were of 37.5% and 18.8%, respectively, and 6.2% were resistant towards ampicillin, streptomycin and tetracycline. None of the strains were resistant towards carbenicillin, cephalothin, ceftriaxone, cefuroxime, gentamicin, kanamycin, nalidixic acid and sulphamethozole.

Plasmid profiling

Of the 16 *R. solanacearum* strains, seven were plasmid-free (RS1, RS2, RS12, RS13, RS14, RS15 and RS16). The other nine *R. solanacearum* strains were classified into two plasmid patterns, P1 harbouring a 7.2 MDa plasmid (RS3, RS6 and RS8) and P2 harbouring a 9.2 Mda plasmid (RS4, RS5, RS7, RS9, RS10 and RS11).

RAPD-PCR fingerprinting

The 70% G + C content primers were chosen to examine the genetic diversity of all *R. solanacearum* strains. The choice of primers used was due to the G + C content of *R. solanacearum,* which was reported at about 67% (Salanoubat et al. 2002). Two primers were identified to produce good separation bands after screening with a subsample of three isolates, to detect polymorphism within *R. solanacearum* strains. The two primers were then tested in subsequent studies with full panel of 16 *R. solanacearum* strains. *Table 1* shows the tabulated profiles of 16 *R. solanacearum* strains from the two primers, P3 (5'-ACGGTGCCTG-3') and P5 (5' GAGATCCGCG-3').

The dendrograms of primer P3 and P5 were constructed as shown in *Figures 1* and *2*. Primer P3 produced fingerprint that could discriminate the *R. solanacearum* strains into four clusters and five single strains at a similarity level of 80% *(Figure 1),* whereas, primer P5 produced three clusters and five single strains at a similarity level of 85% *(Figure 2)*. Two strains (RS3 and RS4) were

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Table 1. Typing of *Ralstonia solanacearum* by antibiotic susceptibility, plasmid profiling, enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and random amplified polymorphic DNA-PCR (RAPD-PCR)

Strain no.	Antibiotic susceptibility pattern[a] and their group	Plasmid (s) size (MDa) and their pattern	ERIC-PCR[b]	RAPD-PCR profiles for primer[b]		Genome types
				P3	P5	
RS1	BPRa (1)	nd	E1	A1	B1	1
RS2	BRa (2)	nd	E2	A2	B1	2
RS3	BRa (2)	7.2 (P1)	E3	ut	ut	3
RS4	nd	9.2 (P2)	E4	ut	ut	4
RS5	BRa (2)	9.2 (P2)	E5	A3	B2	5
RS6	nd	7.2 (P1)	E6	A3	B3	6
RS7	B (3)	9.2 (P2)	E7	A4	B2	7
RS8	nd	7.2 (P1)	E8	A5	B3	8
RS9	nd	9.2 (P2)	E9	A6	B4	9
RS10	nd	9.2 (P2)	E10	A7	B5	10
RS11	B (3)	9.2 (P2)	E11	A7	B6	11
RS12	ND	nd	E12	A8	B6	12
RS13	S (4)	nd	E13	A9	B7	13
RS14	BP (5)	nd	E14	A10	B7	14
RS15	BRa (2)	nd	E15	A11	B8	15
RS16	ABPRaST (6)	nd	E16	A12	B2	16

[a]Tested for ampicillin (A), bacitracin (B), carbenicillin (Car), cephalothin (Cf), ceftriaxone (Cro), cefuroxime (Cxm), gentamicin (G), kanamycin (K), nalidixic acid (Na), penicillin G (P), rifampicin (Ra), streptomycin (S), sulphamethozole (Sxt) and tetracycline (T).
[b]A = Primer pattern for P3; B = Primer pattern for P5; E = Primer pattern for ERIC-PCR
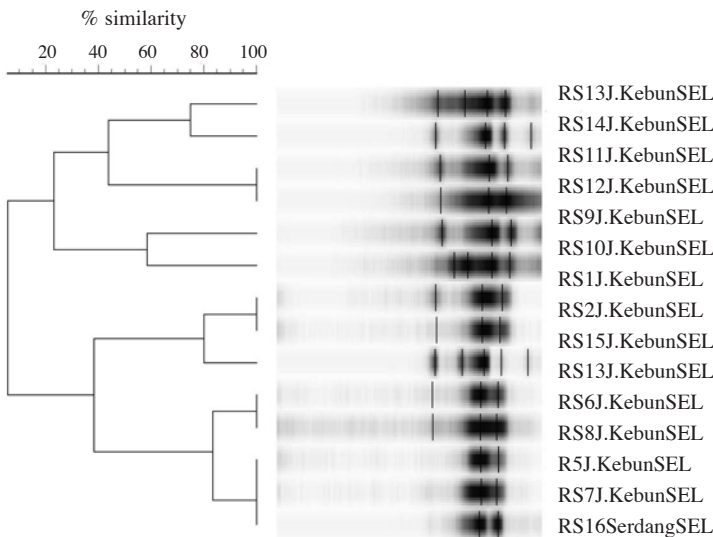ut = Untypable
nd = None detected

*Figure 1. Dendrogram of typable **Ralstonia solanacearum** based on RAPD-PCR patterns by primer P3. RAPD types and geographical locations of each isolate were included*
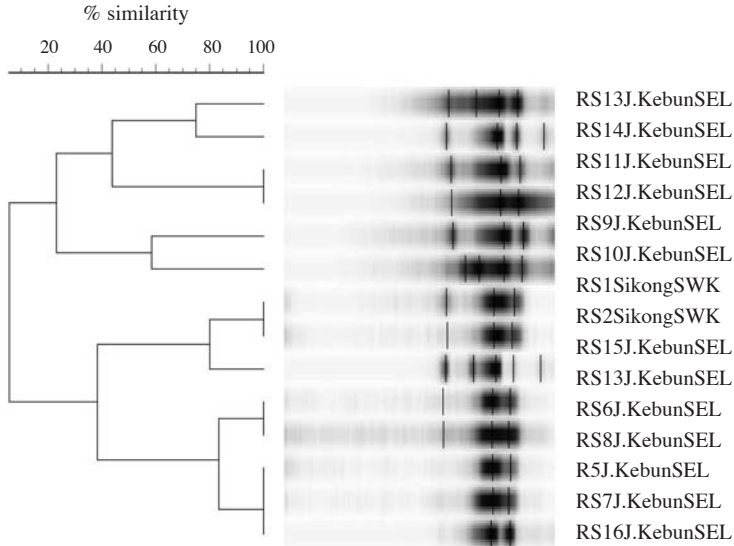
Figure 2. Dendrogram of typable **Ralstonia solanacearum** *based on RAPD-PCR patterns by primer P5. RAPD types and geographical locations of each isolate were included*
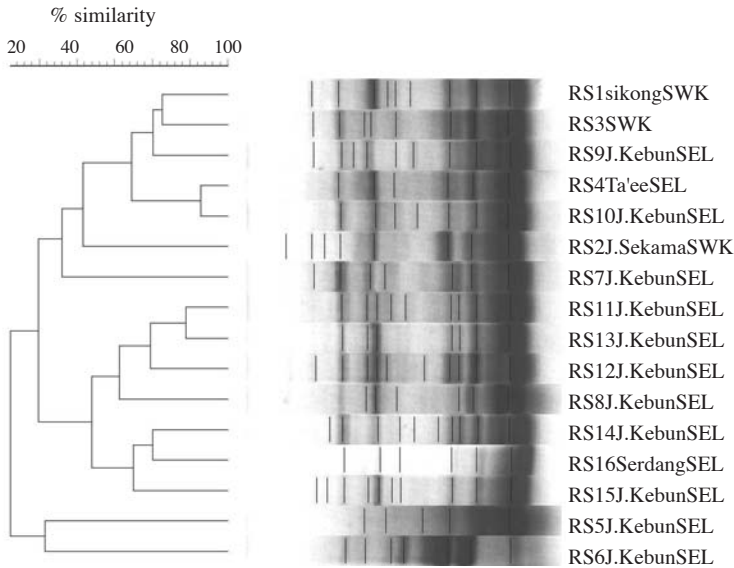

Figure 3. Dendrogram of typable **Ralstonia solanacearum** *based on ERIC-PCR patterns. RAPD types and geographical locations of each isolate were included*

not typable in repeated experiment with both primers.

ERIC-PCR fingerprinting

The dendrogram of typable strains of *R. solanacearum* detected by ERIC-PCR analysis was shown in *Figure 3*. ERIC-PCR produced complex fingerprints which could discriminate all the *R. solanacearum* strains into four clusters and six single strains at a similarity level of 80%.

Discussion and conclusion

Bacterial wilt caused by *R. solanacearum* is one of the most important bacterial plant diseases worldwide. *Ralstonia solanacearum* is a heterogeneous species with a broad host range which exhibits both phenotypic (characters that are expressed by the organism) and genotypic (molecular approaches that involve direct DNA-based analysis of chromosome, plasmid and insertion sequences/transposon) variation (Hayward 1993). To the best of our knowledge, there is a dearth of information on molecular characterization of *R. solanacearum* isolated from tomato plant in Malaysia. In the present study, we examined 16 *R. solanacearum* isolated from tomato plant using antibiotic susceptibility, plasmid profile and the PCR-based methods of randomly amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC).

Antibiotic susceptibility analysis revealed that 10 *R. solanacearum* strains were resistant to a single or more antibiotics and six strains were susceptible to all antibiotics tested. It is obvious that antibiogram or antibiotic susceptibility patterns in the present study, seemed to be less useful to differentiate strains below species level. As indicated in *Table 1,* 10 of the *R. solanacearum* strains can only be classified into six groups according to their antibiotic susceptibility patterns, with another six strains being susceptible to all antibiotics tested.

The results obtained from antibiotic susceptibility patterns is in agreement with the views of Stubbs et al. (1994) who suggested that this method can be used in conjunction with other methods for epidemiological purposes. Therefore, plasmid analysis was conducted as an adjunct technique to increase the discriminatory power in combination with the antibiotic susceptibility. Plasmid profiling analysis is a study to determine numbers and molecular sizes of intact extrachromosomal DNA elements.

However, in this study not all of the *R. solanacearum* tested possessed plasmid. Of the *R. solanacearum* strains examined, seven strains were plasmidless and nine strains possessed a single small plasmid *(Table 1).* Multiple antibiotic resistant and differential susceptibilities to certain antibiotics and harbouring plasmid DNA have been previously reported (Rivera et al. 1991). Thus, the combination of the two methods increased the discrimination of the *R. solanacearum* strains into twelve different groups *(Table 1)*. When compared to the PCR-based techniques used in this study, it was very obvious that the combination of the two methods was less effective in differentiation of *R. solanacearum* strains, although these two methods were easy and relatively cheap to perform.

Due to the speed and simplicity of the protocol involved, the PCR-based arbitrarily primed PCR (RAPD) fingerprinting is among the popular techniques of a new direction in microbiological analysis. Hence in this study, the 16 *R. solanacearum* were examined by the RAPD analysis. A randomly designed 10-mer oligonucleotide set, designated as P1 to P10 was obtained from Genosys Biotechnologies Inc. USA. These primers had a G + C content of 70%. Such G + C content was chosen to maximize the discriminatory power of typing analysis since the G + C content of the genome of *R. solanacearum* was 67% (Salanoubat et al. 2002). Two primers, P3 and P5 yielded clear band patterns, whereas

the remaining primers (P2, P3, P4, P6, P7, P8, P9 and P10) gave bands with only some of the isolates and had poor reproducibility and were not further tested. Such diversities between PCR primers were previously reported by Oakey et al. (1995).

The dendrograms of RAPD-PCR profiles of the 14 typable *R. solanacearum* strains obtained from primer P3 and P5 were shown in *Figures 1* and *2*. As shown in *Table 1,* the DNA products in RAPD-PCR profiles depend on the primer used, with different primers producing different banding patterns. Each primer showed potential of detecting polymorphism between strains. The primer P3 and P5 each detected eight and four RAPD-PCR profiles among 14 typable strains, respectively. Combination of the amplification patterns from the two primers used allowed the identification of 11 RAPD-PCR. Two strains RS3 and RS4 were consistently not typable in repeated experiments. This could be interpreted as the loss of specific sites for primer binding in the chromosomal DNA of these strains. Thus, the RAPD-PCR analysis was considered less effective in distinguishing the difference between *R. solanacearum* strains as compared to ERIC-PCR in this study.

RAPD-PCR has been applied to a number of microorganisms, which allows the discrimination of strains below species level such as *Lactobacillus plantarum* and *L. pentosus* (Van Reenen and Dicks 1996), *Neurospora crassa* (Williams et al. 1990), *Campylobacter jejuni* (Owen et al. 1993), *Listeria monocytogenes* (Mazurier and Werners 1992), *Clostridium difficile* (McMillin and Muldrow 1992) and *Pseudomonas fragi* (Tanaka et al. 1993). The advantages of this method are no prior sequence information about the target is required and primers from a universal set of 10-mer oligonucleotides can be used. This makes it a tool of great power and general applicability.

The enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) or rep-PCR is now being widely used for phylogenetic analysis and differentiation of bacterial strains. This rep-PCR targets the interspersed repetitive DNA elements (referred to as 'rep') that are located in different positions and are separated by various distances depending on the bacterial species or strains. The PCR amplification of the genomic region between these repeat elements will yield DNA fragments of various sizes, depending on the length of DNA that exists which requires no previous knowledge of nucleotide sequence.

The results obtained in the present study showed that ERIC-PCR was highly discriminating in distinguishing the 16 *R. solanacearum* strains into 16 distinct ERIC-PCR profiles. *Figure 3* shows the dendrogram of typable *R. solanacearum* strains by ERIC primer. Thus, the isolates examined in ERIC-PCR analysis exhibited high level of local geographical genetic variation. This finding is in agreement with Julian et al. (1995) who reported on the genetic diversity of *R. solanacearum* observed in race 3 in Kenya.

In this study, the homogeneity in the antibiotic resistant patterns and plasmid profiles (with many strains sharing the same patterns) made it difficult to perform epidemiological observation and trace the distribution of *R. solanacearum* in the different locations. Though the level of discrimination obtained with RAPD-PCR was high, its inability to type two strains has made it impossible to make a complete comparison on all the *R. solanacearum* strains examined. The ERIC-PCR profiles of *R. solanacearum* demonstrated their genetic variety and the close genetic relationship between strains found in Sarawak and Selangor. For example, RS4 from Sarawak and RS10 from Selangor had more than 93% genetic similarity. The recovery of closely related strains such as the RS4 and RS10 from two areas separated by the South China Sea may direct an investigation towards establishing the vehicle of

transmission of this plant pathogen between the two very distant locations.

In conclusion, the results of this study have indicated that PCR-based fingerprinting may be useful in studying the epidemiology of the bacterial wilt pathogen, *R. solanacearum.*

Acknowledgement

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Abstrak

Ciri-ciri epidemiologi *Ralstonia solanacearum* yang diasingkan daripada pokok tomato di Sarawak dan Selangor telah dikaji. Analisis epidemiologi strain telah dikaji melalui kerintangan terhadap antibiotik, profil plasmid dan kaedah RAPD serta ERIC. Enam strain mudah dipengaruhi dengan semua antibiotik yang telah diuji, manakala 10 strain lain yang rintang terhadap satu atau dua antibiotik telah dikumpulkan kepada enam corak kerintangan antibiotik. Plasmid tunggal yang kecil, bersaiz 7.2 Mda dan 9.2 Mda telah didapati di kalangan sembilan strain yang mengandungi plasmid dan membolehkannya dikumpulkan kepada dua corak plasmid.

Dalam kaedah dasar PCR menggunakan RAPD dan ERIC, dua strain tidak dapat ditaipkan dengan menggunakan kaedah RAPD manakala kesemua strain boleh ditaipkan dengan menggunakan kaedah ERIC. Dalam kajian ini, kepelbagaian strain *R. solanacearum* yang tinggi telah dikaji. Analisis ERIC menunjukkan kaitan klon antara pencilan daripada pokok tomato di Sarawak dengan di Selangor. Kehadiran *R. solanacearum* yang serupa pada pokok tomato dari dua tempat yang berjauhan lokasi perlu dipertimbangkan jika keputusan dari strain tomato capjari boleh digunakan untuk mengesan cara perpindahan mikroorganisma tersebut.