

Improving a PCR-Based Method for Identification of *Ralstonia solanacearum* in Natural Sources of West Malaysia

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Abstract: *Ralstonia solanacearum*, a soil-borne plant pathogen, causes lethal wilting disease of more than 200 plants worldwide. This very wide host range covers both monocots and dicots, extending from annual plants to trees and shrubs. Although generally it's considered as a plant pathogen, *R. solanacearum* behaves primarily as a saprophytic bacterium able to survive for long periods of time in various natural habitats such as surface waters and different types of soils. Epidemiological and ecological studies on pathogen distribution in the environment are seriously hindered by the lack of efficient detection method especially when the concentration of the pathogen is either very low or is present in a latent, dormant or non-culturable state. With due attention to importance of *R. solanacearum* in Malaysia and several irreparable losses that every year caused by this bacterium, this is prompted current study to develop a sensitive PCR-Based method to improve the detection of *R. solanacearum* in natural sources. We selected the previously reported primers (OLI1; OLI2; Y2; JE2) for their sensitivity and specificity detection of the bacterium in water and soil by a modification of PCR assay.

Key words: *Ralstonia solanacearum*, bacteria, PCR and West Malaysia

INTRODUCTION

Ralstonia solanacearum, previously known as *Pseudomonas solanacearum* (Smith, 1896), in the Proteobacteria β subdivision is a plant pathogenic bacterium commonly found in the soils of tropical and subtropical countries where it devastates cultivation of many crop plants^[1,2]. This organism, responsible for bacterial wilts, can infect over 300 plant species belonging to over 30 botanical families^[3]. Major agricultural hosts include tobacco, tomato, potato, eggplant, chili and banana trees^[4]. Weeds appear as alternative hosts for the pathogen to survive in the absence of its susceptible host plants^[3]. Field symptoms of this bacterium are rapid and irreversible wilt under favorable conditions, stunting and yellowing of foliage^[1]. Epidemiological and ecological study of *R. solanacearum* is difficult because the pathogen has an extremely broad host range and is able to survive in the soil in the absence of the host plant^[1]. Moreover, it can colonize host plants like members of the Solanaceae and non-host plants including many weeds,

without producing any visible symptom^[3]. *R. solanacearum* can persist at low populations in naturally infested soil for years without a host plant and the population size could increase to the plant infection threshold within a season after the host plants are returned to the fields^[3]. Therefore, early detection of the bacterium in soil, water, weeds, tubers and plant residues could facilitate elimination and certainly reduce the risk of crop loss. However, the commonly used methods such as isolation on semi-selective medium, serological methods (ELISA or immunofluorescence), or pathogenicity tests on host plants for the diagnosis of bacterial wilt are often inadequate in terms of specificity, sensitivity or response time, especially for detecting the bacterium in soil and water^[5]. In order to optimize the efficiency of prophylactic measures, powerful tools for the identification and detection of the bacterium in diverse substrates (plant, seed, water & soil) are required. DNA amplification pathogen offers many advantages over classical techniques; neither purification nor cultivation of the pathogen are required and the specificity,

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sensitivity and response time of tests are improved. Nevertheless, the PCR method has not yet become a routine diagnostic tool for many laboratories, mainly because of the inhibition of the amplification reaction by compounds contained in crude bacterial extracts, which give false negative results or low detection sensitivity^[6,7,8]. Until now there is a very few validated PCR protocol recommended for the detection of *R. solanacearum* in samples especially in soil and water samples. Therefore, this study was to develop and compare several procedures to overcome PCR inhibition problems and to propose a set of standard protocols for reliable detection of *R. solanacearum* whatever the origin of the sample.

MATERIALS AND METHODS

Bacterial sample collection: Field soil samples used for this study were collected from banana, tomato, chili, eggplant and tobacco fields in West Malaysia. Samples were collected randomly, at a depth of 5 to 30 cm, to form a composite sample for each unit^[9]. At each unit, 10 g of soil were taken to make a 100 ml-soil suspension for detection. Irrigation water samples were collected from same fields or from water canals in same area. Water samples were collected (50 mL per sample) at the entrance and exit spots of the irrigation ditches for each field. All field samples were maintained at 4°C in plastic bags during transportation and storage before testing.

Improved bacterial DNA extraction: All soil samples were crushed and suspended by blender using Tris buffer (100 mM). One milliliter of supernatant from each suspension (and water samples directly) was added to 9 mL of liquid SMAS medium (SMSA without agar). The cultures was incubated at 30°C with 150 rpm for 24 h. After enrichment, 5 mL of enriched cultures were placed in 200 mL-tubes and placed into a boiling water bath for 5 min. After a low speed centrifuge (1500 rpm, 5 min), the supernatant was transferred to a new microfuge tube and equal volume of phenol:chloroform:isoamyl alcohol (25:24:1; pH 7.6) was added, which they were mixed by vortexing at the maximum speed for 1 min and centrifuged at 16000 g for 10 min. The aqueous phase was transferred to new tube and 0.1 volume of 3M sodium acetate (pH 5.2) and 0.6 volume of isopropanol were added. The solution was mixed well and centrifuged at 16000g for 15min. DNA pellets were washed with 70% ethanol twice, dried and resuspended in 20 µL of TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer.

Two microliter of this DNA solution were taken for PCR analysis. To compare the quality of this method with other methods, DNA extraction were performed for all samples using two other methods that already have been described by Elphinstone *et al.*^[7] and Pradhanang *et al.*^[9].

PCR amplification: The reaction was preformed in final volume of 25 µL using primer pairs OLI-1/OLI-2 followed by Y2/JE2 according to nested PCR procedure detailed by Poussier and Luisetti^[10]. Moreover, 0.5 ng µL⁻¹ of BSA (Bovine serum albumin) were added to PCR mixture to prevent any inhibition effect on PCR. PCR products were analyzed by electrophoresis on 1% agarose gel and visualized UV light (300nm) after Ethidium Bromide staining. Also conventional PCR was preformed with OLI-1/Y2 and JE2/OLI-2 according to Seal *et al.*^[11] and Elphinstone *et al.*^[6] respectively.

Evaluation sensitivity of improved detection method in natural samples: To determine the detection threshold of our improved method and compare it with other methods, bacterial suspension of *R. solanacearum* strain were prepared by making a 10-fold dilution series from a liquid culture. Two bacterial strains that already had been confirmed as *R. solanacearum* were used for this experiment. Soil samples were spiked with these two strains by mixing each 100 g autoclaved soil with 10 mL of bacterial suspensions each containing from 10¹ to 10⁸ CFU mL⁻¹ bacteria. The inoculated soil samples were incubated at 28°C for 24h prior testing. To determine detection threshold of *R. solanacearum* in water samples, 500 mL of autoclaved irrigation water were artificially contaminated by same serial dilutions of *R. solanacearum* strain, giving a final concentration from 10¹ to 10⁸ CFU mL⁻¹ then the improved DNA extraction method were applied to all samples. Before DNA extraction all sample were infested by some bacterial isolates that had been grown on TZC medium but have not been confirmed as *R. solanacearum*.

RESULTS AND DISCUSSION

Sample collection: A total of one hundred soil and water samples were collected from all parts of West Malaysia. The sampling sites were included vegetable farms and other production areas planted with known hosts of the pathogen, such as banana and tobacco. The presence of *R. solanacearum* in the soil or water of these areas already had been confirmed by Khakvar *et al.* [unpublished data].

PCR amplification: Both of the PCR methods (conventional and improved Nested-PCR) could detect *R. solanacearum* strains in samples. In our improved nested-PCR, in first step, a 410 bp amplicon was produced. Followed PCR (nested) produced a 220 bp amplicon in sixty-nine samples. No other bands were observed in all samples. Conventional PCR with primers OLI-Y2 produced 280 bp amplicon in 60 samples while a very weak band (90 bp) was observed in 12 of these samples. PCR with primer JE2-OLI produced a 340 bp amplicon in 67 samples while in two of these samples two weak bands (48 and 390 bp) were observed. In 23 samples, no band was shown by both methods (Fig. 1).

Sensitivity threshold: The sensitivity of the individual PCR using the OLI1/OLI2/Y2/JE2 primers were measured with 10-fold dilution series of *R. solanacearum* samples that artificially were contaminated. The bacterium was detectable in samples as few as 10^2 - 10^5 CFU mL⁻¹ in nested PCR without any non-target band. The sensitivity of detection with conventional PCR using OLI1/Y2 & JE2/OLI2 nearly was same (10^4 - 10^5 CFU mL⁻¹) but some non-target bands were observed especially in soil samples (Table 1).

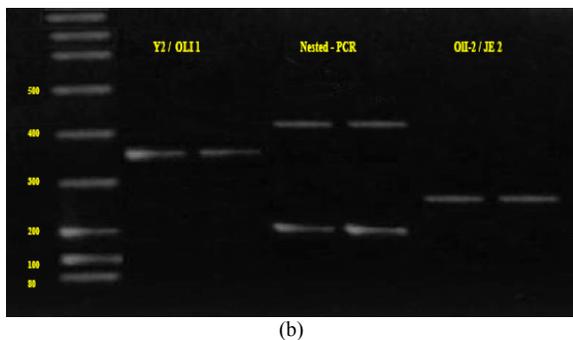
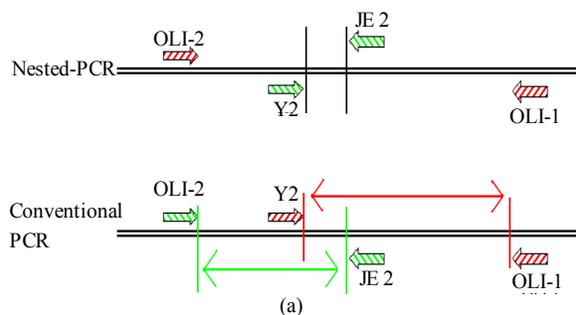


Fig. 1: (a): The primers with their positions that have been used for conventional and nested-PCR, (b): Results of conventional and nested-PCR

Table 1: Sensitivity and specificity of PCR reactions in *R. solanacearum* samples

	Sensitivity in soil sample	Sensitivity in water sample	Non-target band in positive samples	Non-target band in negative samples
Nested-PCR	10^3 - 10^5	10^3 - 10^5	-	-
OLI/Y2	10^4 - 10^6	10^4 - 10^6	+	-
JE2/OLI2	10^4 - 10^6	10^4 - 10^6	+	-

The results presented above demonstrate the development of an improved PCR-based assay capable of detection and identification of *R. solanacearum* directly in natural sources. In routine laboratory studies, *R. solanacearum* can be detected by conventional PCR assays in aqueous suspensions ranging from 10^3 CFU mL⁻¹ by the method of Seal *et al.*^[11] to 10^5 CFU mL⁻¹ by the method of Elphinstone *et al.*^[7]. However, in plant samples, detection limit for former assay have been shown that can be increased to 10^6 CFU mL⁻¹ or higher due to low concentration of PCR inhibitor substances^[12,13,14]. Previous studies have been shown that primers of OLI1, OLI2, Y2 and JE2 are not completely specific for species of *R. solanacearum* but nested-PCR using with these primers provide a very specific tool for differentiation of *R. solanacearum* strains in soil and water samples. Close detection threshold of conventional PCR and nested-PCR in this study shows that providing a suitable method for DNA extraction from samples is the first and the most important part of a reliable detection of Bacteria in samples. Bacterial DNA extraction in this study is shorter and more easier than previous methods^[15], therefore, this method can be used as a sufficient and reliable method for direct detection of *R. solanacearum* in water and soil samples.

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