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Molecular identification of *Ralstonia pseudosolanacearum* causing bacterial wilt in tomatoes from Da Nang by using Colony PCR

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ABSTRACT

Ralstonia solanacearum species complex (RSSC) is the main pathogen causing bacterial wilt disease in tomatoes. This study applied the colony polymerase chain reaction (PCR) technique to rapidly screen and select RSSC strains from isolated bacteria of diseased tomatoes. This method directly used bacterial colonies on the Petri plate as templates to amplify with RSSC's specific and multiplex primers. The results showed that the Vietnamese isolates were identified as R. pseudosolanacearum, phylotype I. Phylogenetic analysis of the 16S-23S rDNA sequencing also confirmed these results. Therefore, this is the first report to recognize R. pseudosolanacearum phylotype I as the cause of bacterial wilt disease in tomatoes from Danang.

1. INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* species complex (RSSC) presents a serious threat to agricultural production by affecting more than 200 hosts in 54 botanical families (Prior et al., 2016). The host range of *R. solanacearum* includes many important crops such as tomato, potato, eggplant, tobacco, peanut, and banana, leading to both severe economic and social impacts (Hayward, 1991; Denny, 2007). In addition, this disease is seen worldwide, yet devastating in the tropics and subtropics (Hayward, 1994; Paudel et al., 2020). Until now, the RSSC strains were divided into four phylotypes (I, II, III, and IV) by using specific multiplex PCR. The RSSC strains belonging to phylotype I are mostly of Asian origin, for phylotype II strains are from the Americas (Sharma et al., 2022). Phylotype III are strains mainly found in Africa and surrounding islands (Sharma et al., 2022). Finally, RSSC belongs to phylotype IV, which includes strains from Indonesia, Japan, Korea and Australia (Fegan and Prior 2005; Jeong et al., 2007). Based on DNA-DNA hybridizations and phylogenetic analysis, RSSC was classified by three genospecies system consisting of *R. pseudosolanacearum* (phylotypes I and III), *R. solanacearum* (Phylotype II), and *R. syzygii* (phylotype IV) (Safni et al., 2014).

In terms of control of the disease, there have been many measures to prevent the causative agent of bacterial wilt diseases such as cultivation, soil improvement or the use of plant protection chemicals. Research on crossbreeding resistant varieties to control pathogens has so far not yielded any significant results (Vu et al., 2017; Tian et al., 2021). More importantly, misuse and overuse of chemical agents or bactericides could give rise to agricultural soil pollution and place pressure on the ecological environment. Therefore, it is necessary to determine timely and accurately the state of

bacterial wilt disease and its spatial distributions in order to reduce the infection by biological control or even early removal of the infected plants (Chavez et al., 2012). The use of previous wilt pathogen detection methods such as field inspection or manual differentiation, and biochemical methods are often time-consuming and labor-intensive. Yet, most of these methods are also not very effective. In addition, the use of biochemical methods for early detection of pathogens also seriously affects the environment (Xie et al., 2015). Since disease control methods are not effective, it is necessary to diagnose the causative agent to find treatment. Therefore, up to now, a number of studies on methods for detecting and diagnosing the causative agent of green wilt have appeared (Cen et al., 2022), including enzyme-linked immunosorbent assay (ELISA) (Rajeshwari et al., 1998), and especially polymerase chain reaction (PCR) with specific primers (Fegan & Prior, 2005). A method for screening individual bacterial colonies by PCR to obtain the specific DNA sequences has been developed (Kramer & Coen, 2001). This procedure, commonly referred to as colony PCR, provides a quick and easy way to screen a large number of bacterial colonies without DNA extraction (Woodman, 2008). Recently, colony PCR of *R. solanacearum* has been performed in a number of studies with 16S rRNA primers and species-specific primers (Umesha et al., 2012; Prakasha, 2016).

In Viet Nam, RSSC has been reported (Truong et al., 2018), but the use of the 16S - 23S rRNA region to classify RSSCs has not been studied. Therefore, in this study, RSSC isolates from Da Nang city were evaluated according to recent classification systems, and colony PCR to detect RSSC.

2. MATERIALS AND METHOD

Sample collection and bacterial isolation

Tomatoes with bacterial wilt symptoms were collected from Hoa Vang district, Da Nang city, Viet Nam. A knife or scissors were used cut trunk segments (10 cm) at sites of symptomatic tissue to ensure that the original sample contained the causative agent. Next, clamps were used to place the disease sample into a zip bag, which was then sealed, and a sample number is given. Samples were then brought to the laboratory to isolate pathogenic bacteria.

Stem samples were washed with double-distilled water (ddH_2O), and the surface was disinfected with 70% ethanol, soaked for 5 minutes in sterilized ddH_2O, and then the fluid inside the stem was collected by the technique of Longchar et al. (2020). Thereafter, 100 µl stem fluid was cultured on a Petri plate containing TZC medium (1% peptone; 0.5% glucose; 0.1% Casein hydrolysate 0.05% 2, 3, 5 – Triphenyl tetrazolium Chloride (TTC); 0.5mg Penicillin; 1.7% Agar; pH=7-7.2) (French et al., 1995; Chaudhry & Rashid, 2011). After 48 hours of culture, colony morphology on Petri plate was observed. Bacterial colonies having the morphology as RSSC were selected for further study.

Colony PCR and method of DNA extraction

For colony PCR studies, colonies were collected by an inoculation loop from Petri plates, then diluted in 10 µL of sterilized ddH_2O. PCR reactions were performed with 759/760, multiplex PCR, and 16S-23S rRNA region primers for RSSC identification, phylotype, and three genospecies determination, respectively (Table 1). The total volume was 20 µL containing 1X MASTER MIX (PHUSA Biochem, Vietnam), 1µL of colony solution, 1pmol/µL for each primer (Table 1), and ddH_2O. Thermal cycles for each primer set followed those of previous studies (Opina et al., 1997; Fegan & Prior 2005; Li & Boer 1995). PCR products were electrophoresed on 1% agarose gel in 1X TAE buffer at 100V for 30 min. Electrophoresis results were observed on a Major Science UVDI instrument. Finally, PCR products were sent to First BASE Laboratories Sdn Bhd (Malaysia) for sequencing.

Phylogenetic analysis

71 sequences of 16S-23S rDNA gene region were collected from the GenBank (https://www.ncbi.nlm.nih.gov/). The sequences were then aligned using ClustalW (Thompson et al., 1994; Kumar et al., 2016). Due to a large number of sequences, phylogenetic trees were constructed using the neighbor-joining (NJ) clustering method, and evolutionary distances were computed using the p-distance method (Saitou & Nei, 1987; Nei & Kumar, 2000). Bootstrapping with 1000 replicates was adopted to enhance confidence (Felsenstein, 1985). All the above was analyzed in MEGA 7 (Kumar et al., 2016). The phylogenetic trees were then edited and noted by Interactive Tree Of Life (iTOL) (Letunic & Bork, 2021).

Table 1. Primers used in multiplex PCR

Primer	Primer of sequence (5'-3')	Size (bp)	Specificity	References
759	GTCGCCGTCAACTCACTTTCC	282	RSSC	Opina et al., 1997
760	GTCGCCGTCAGCAATGCGGGAATCG		Species-specific	
Nmult: 21:1F	CGTTGATGAGGGCGCGCAATTT	144	Phylotype I	Fegan & Prior, 2005
Nmult: 21:2F	AAGTTATGGACGGTGGAAGTC	372	Phylotype II	
Nmult:22: InF	ATTGCCAAGACGAGAGAAGTA	213	Phylotype IV	
Nmult:23:AF	ATTACGAGAGCAATCGAAAGATT	91	Phylotype III	
Nmult:22: RR	TCGCTTGACCCTATAACGAGTA			

3. RESULTS AND DISCUSSION

Eight colonies morphologically similar to RSSC were collected. RSSC forms colonies with an irregular shape, mucilage, a white margin, a pale pink tinge in the center, rounded colonies, a red center, and a small outer border. The morphology of the collected colonies is described in detail in Table 2. These colonies were generally round in shape, with mucus, pale pink, or red pink (Figure 1).

Classification of RSSC isolate in Da Nang city

After performing colony PCR with specific primers 759/760, colonies 1 and 8 showed band lines with product sizes about 300 and 1000 bp, respectively (Figure 2). No band were observed in the remaining colonies. Thus, only colony 1 showed the band with similar size to RSSC (Table 1). After that, the 16S-23S rRNA region of strain colony 1 (T04) was amplified, sequenced, and analyzed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The result btained from BLAST showed that this strain belonged to RSSC (Table 3). The sequence of strain T04 has been submitted to GenBank with accession number: OP591394.

Next, we selected 10 colonies of strain T04 to perform colony PCR with a Multiplex primer set. The results showed that all colonies displayed two bands on the agarose gel. The band sizes were between 140bp and 300bp (Figure 3). The upper band could be determined as PCR product of 759/760 primer, while the other band proved that RSSC strain T04 was R. pseudosolanacearum and belonged to Phylotype I (Table 1). We used 10 sub-colonies of colony 1 for the purpose of verifying the effectiveness of the application of colony PCR technique with multiplex primer sets. The results obtained were very positive, with an efficiency rate of the reaction up to 100%.

Table 2. The morphology of the colonies selected PCR technique with primer pairs 759/760

Collected colonies	Strains	Colony morphology
Colony 1	T04	Round in shape, smooth surface with a pink center, a white mucus border around it, and quite large
Colony 2	TT	Similar shape to colony 1, round shape, smooth surface, pink center with white mucus border around but smaller size.
Colony 3	TT1	Round, small in size, with a red center and a large surrounding white border
Colony 4	TF	Round, large in size, smooth surface with a dark red center and a small surrounding white border
Colony 5	TF2	Round, quite small in size, with a red center, a white center in the center, and a small white border around it.
Colony 6	TS	Round, quite small in size, smooth surface pink in center, white in center, and small around white border.
Colony 7	TS2	Similar shape to colony 4, round shape with dark red center, small white border around and much smaller size.
Colony 8	TE	Similar shape to colony 6, round shape with smooth surface with pink center, white center with small surrounding white border and much smaller size.

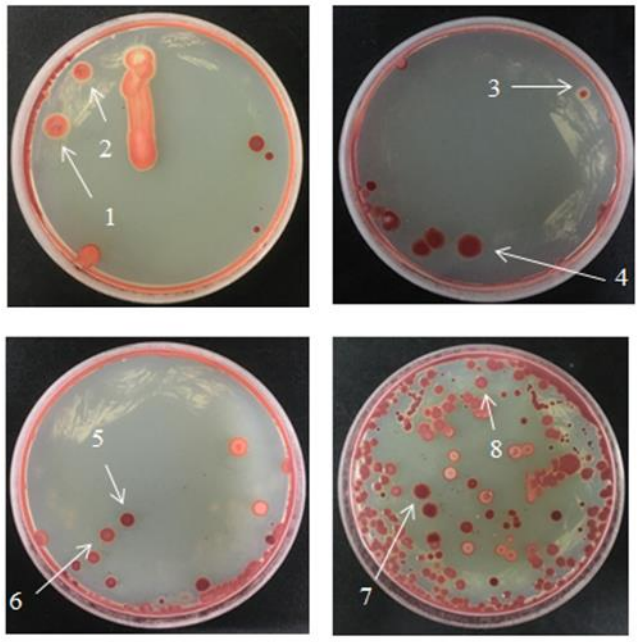

Figure 1. Isolated bacteria from infected tomato plants on TTC medium. The arrows indicate colonies morphologically similar to RSSC strains (Table 2)

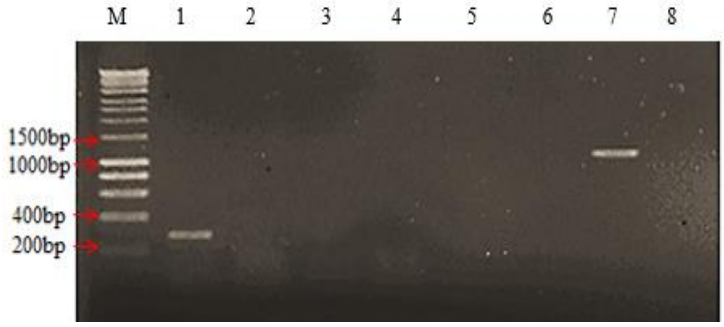

Figure 2. Colony PCR results obtained from eight collected colonies using primer pair 759/760

M: Ladder 10kb; Lane 1: colony 1; Lane 2: Colony 2; Lane 3: Colony 3: Lane 4: Colony 4; Lane 5: Colony 5; Lane 6: Colony 6; Lane 7: Colony 7; Lane 8: Colony 8

Table 3. Sequences producing significant alignment with RSSC strain colony 1 (T04)

Name of sequence	Max score	Total score	Query cover	E-value	Percent of identification
Ralstonia solanacearum strain ZCz-2	1092	1092	100%	0.0	100.00%
Ralstonia solanacearum strain FJAT1303.F1	1088	1088	99%	0.0	100.00%
Ralstonia solanacearum strain FJAT1303.F1	1088	1088	99%	0.0	100.00%
Ralstonia solanacearum strain FJAT1303.F50	1088	1088	99%	0.0	100.00%

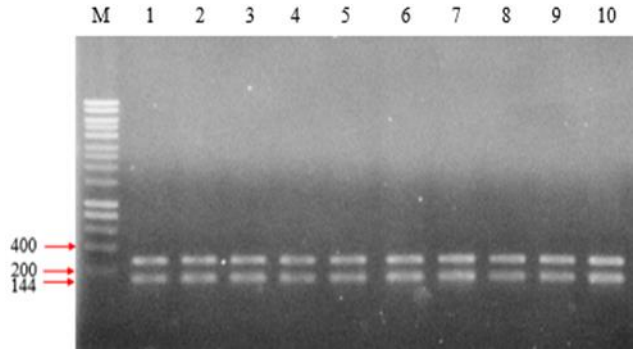

Figure 3. Colony multiplex PCR obtained from 10 colonies of *R. pseudosolanacearum* strain T04 using species-specific and phylotype specific primers

M: Ladder 10kb; Ctrl: Positive control; Lane 1-10: Ten sub-colonies of Colony 1.

Phylogenetic analysis

Sequences of 16S–23S rRNA regions of 71 RSSC strains were collected from the GenBank to evaluate the relationship between the Vietnamese strain and other strains worldwide. The results showed that RSSC strains can be divided into three main branches corresponding to three genospecies: *R. pseudosolanacaerum*, *R. solanacacearum*, and *R. syzygii* (Figure 4). *R. pseudosolanacaerum* included two clades as phylotypes I and III. The Vietnamese strain T04 belonged to the phylotype I clade, which was similar to the results obtained from Multiplex PCR. Most phylotype I strains originate from Asia and are closely related with a bootstrap coefficient: 90-100% (Figure 4).

Tomatoes showing symptoms of bacterial wilt were observed in Da Nang city, Viet Nam. Bacteria were isolated from the stem of diseased tomatoes on TZC medium. Eight obtained strains had morphology similar to RSSC which was described in previous studies (Kelman, 1954; Hayward, 1994; Longchar et al., 2020). These strain colonies owned round shape, slimy, white margin, pale pink or red pink in the middle (Figure 1, Table 2). By relying on the characteristic morphology of *R. pseudosolanacearum* on TZC medium, we have excluded some colonies, whose morphology is not pathogenic.

Next, we used colony PCR technique with primer pair 759/760, striped product with the size of about 280 bp was found. This result is similar to the study of Rado (2015). identification of colony morphology combined with colony PCR, helped to isolate and quickly detect *R. pseudosolanacearum* bacteria. Furthermore, in this experiment we also tested the efficiency of colony PCR with 10 colonies that are offspring of colony 1. The results show that the colony PCR technique is suitable with RSSC to confirm quick identification. After that, continue to perform colony PCR with primer 16S - 23S and sequence the gene, the results were compared on the GenBank of the bacterium. This is the first study in Viet Nam using 16S–23S rRNA region sequences to identify strains of RSSCs. From the results, the bacteria isolated from the diseased tomato was *R. pseudosolanacearum*. In addition, from investigating the efficiency of colony PCR reaction with Multiplex, it can be stated that this method gives PCR products with the same size and position as when using extracted DNA. The band line is similar in size to the Multiplex primer set for subspecies assessment (Table 1). The phylogenetic tree structure coincided with previous studies (Fegan and Prior 2005; Jeong et al., 2007; Safni et al., 2014). *R. pseudosolanacearum* strain T04 was found to be a close relation with other Asian strains such as whose found in China, Indonesia, Taiwan, Japan, and India (Figure 4). Our results suggest that the use of colony PCR method is effective not only with 1 pair of 759/760 primers, but also can be applied to multiplex PCR reactions. This allows for improved efficiency, reduced time and cost savings in the detection and identification of RSSCs.

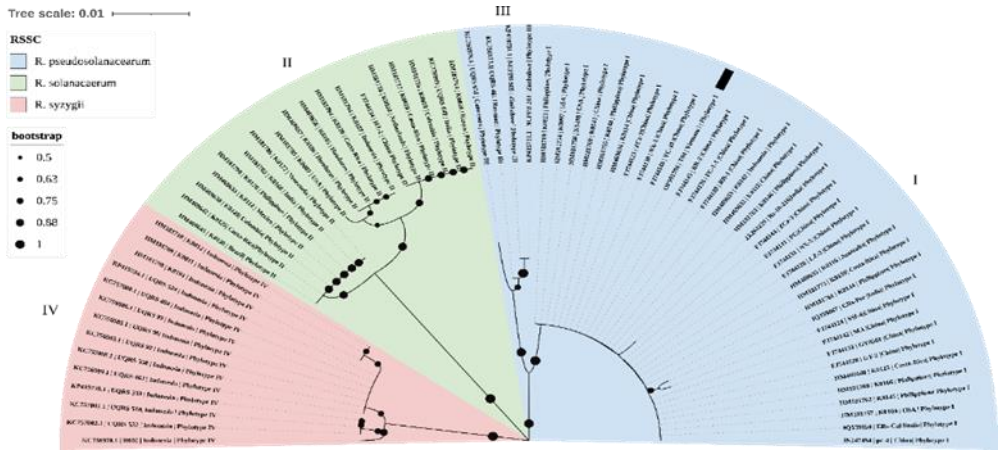

Figure 4. Neighbor-joining phylogeny derived from 16S–23S rRNA gene regions of RSSC strains

4. CONCLUSION

In this study, we identified the bacterial strain causing bacteria wilt on tomato plants in Da Nang as belonging to the species *Ralstonia pseudosolanacearum*, phylotype I. Besides, the colony PCR proved effective in identifying this bacterium and provides a rapid pathogen diagnosis protocol.

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