

“Research Note”

Application of Specific Primers and Polymerase Chain Reaction for Identification of *Ralstonia Solanacearum* Biovar2

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ABSTRACT- A rapid and sensitive polymerase chain reaction (PCR) method is described for the determination of latent infections of *Ralstonia solanacearum*, the causal agent of bacterial wilt of solanaceous plants. The PCR tests with specific primers successfully detected *R. solanacearum* biovar 2 in all naturally and artificially infected potato samples with or without visible symptoms, and gave a characteristic 1019 bp band with division 2 specific DIV2F / DIV2R primers. As expected the PCR test with division 1 specific DIV1F / DIV1R primers did not produce any PCR product with any infected potato and tomato sample. None of the healthy control potato and tomato plants gave visible PCR products with the specific primers.

Keywords: *Ralstonia solanacearum*, Bacterial wilt, PCR, rDNA

INTRODUCTION

The bacterial wilt disease of solanaceous crops caused by *Ralstonia solanacearum* (24) is one of the most important diseases in tropical and subtropical regions of the world. *R. solanacearum* is a heterogenous species divisible into several races and biovars based on host range, pathogenicity (4, 5) and metabolic versatility (11). This organism infects a wide range of plants including potato, tomato, tobacco, eggplant, peanut, banana, pepper, dahlia, marigold, nasturtium, zinnia and many common weeds (11, 13).

R. solanacearum strains can infect potato, tomato, banana and ginger without developing any symptoms. Therefore, a method for the detection of latent infection of planting materials is desirable. Latently infected planting materials are important shelters for long term survival and spread of *R. solanacearum*.

Bacterial wilts of potato and banana have spread locally and internationally on latently infected potato tubers and banana suckers (1, 8, 14). Latently infected tubers may produce heavily infected plants and hence spread the pathogen into previously noninfested soil (14). Latent infection is also an important problem in breeding programs in which progenies are screened at seedling stage by root inoculation and the tubers that are generated several months later by resistant plants may carry the bacterium (7).

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Seal *et al.* (18, 19) and Taghavi *et al.* (21) reported the construction of specific primers based on partial and complete sequencing of 16S rDNA of *R. solanacearum*, *R. syzygii* and blood disease bacterium of banana (BDB), which made feasible the detection of *R. solanacearum* isolates in culture and planting materials by polymerase chain reaction (PCR) amplification. Boudazin *et al.* (6), Weller *et al.* (22) and Poussier *et al.* (15) also described PCR-based methods and specific primers for detection of *R. solanacearum* biovars. The primer pair DIV2F + DIV2R amplifies *R. solanacearum* strains of division 2 including biovars 1, 2 and N2 and also *R. syzygii* and BDB. The DIV1F + DIV1R primer pair is specific for division 1 strains of *R. solanacearum* including biovars 3, 4 and 5 and aberrant strain of biovar 2 (18, 21).

Bacterial wilt of potatoes was originally reported from Isfahan province, Iran in 1988 (3). It was later found in other parts of Iran and was attributed to biovar 2 and race 3 of *R. solanacearum* (2).

In this study, the application of specific primers and PCR for the detection of latent infection of *R. solanacearum* in potato and tomato without prior isolation and enrichment of pathogen from plant tissues was evaluated.

MATERIALS AND METHODS

Bacterial strains Strains of *R. solanacearum* used in this study were isolated from potato and identified as biovar 2 (2). Isolate ACH158 (from Dr. A. C. Hayward, Department of Microbiology, The University of Queensland, Australia) was used as a reference strain. The stock culture suspensions of bacterium in sterile distilled water were streaked on sucrose peptone agar (SPA) plates and incubated at 28°C for 48 h, to obtain fresh cultures (10).

Plants Healthy potato tubers of the cultivar Agria and tomato variety Raklode (from Agricultural Research Station, Hamadan Province, Iran) both susceptible to *R. solanacearum* were used for inoculations.

Inoculation Potato tubers were inoculated with five isolates of biovar 2 either with bacterial suspension adjusted to $A_{600} = 0.1$ (about 10^8 cells/mL) (12) or with sterilized tooth picks dipped in growing colonies on agar. Tubers were planted in plastic pots containing steamed soil and grown in the growth chamber at 18°, 23° and 28°C with a photoperiod of 12 hrs. Four pots, containing two tubers per strain and per temperature including the control, were inoculated. Potato plants were examined at weekly intervals for bacterial wilt symptoms. Tomato seedlings were grown in sterilized soil in plastic pots and placed in the same growth chamber condition. The stems of seedlings were inoculated with 5 isolates of biovar 2 at two-leaf stage with sterilized tooth picks dipped in the growth culture. Control potato and tomato plants were inoculated with sterilized distilled water. All potato plants were kept in a greenhouse until they generated daughter tubers and were harvested 45-75 days after inoculation except for those plants at 28°C

which were harvested 14 days after inoculation. The tomato plants were sampled 7-14 days after inoculation.

Preparation of samples for PCR.

Tubers Potato tubers harvested from the inoculated and control plants were washed with water to remove surface soil and a cone-shaped plug was cut from the stolon end with a sterilized blade. The holes were flooded with 200-500 μ L of sterile distilled water depending on the size of the hole cut water was then transferred from the holes to 1.5 mL centrifuge tubes after 1-3 min. Samples were centrifuged for 5 min at 12000 g and the supernatant was removed carefully leaving 50-100 μ L of the sample in the tube. The pellets were resuspended and vortexed in 50-100 μ L of the left supernatant and one loopful of sample was streaked on SPA plates. The resuspended pellets were boiled for 10 min in 0.5M NaOH (final concentration) to reduce the effect of phenolic inhibitors and allowed to return to room temperature before their use in PCR.

Stems Stem of potato and tomato plants were washed with water, cut with a sterilized scalpel near the base and were placed in sterile distilled water for about 10 min to allow bacteria to ooze out. The bacterial suspensions were concentrated by centrifugation as described above and the supernatant was discarded leaving 50-100 μ L to avoid disruption of the pellets. The pellets were resuspended and plated as before or further used in PCR.

Polymerase Chain Reaction. PCR amplifications were performed using a Master Cycle 5330 thermal cycler. The reaction mixture for both primer pairs contained 2.5 μ L of 1 \times PCR buffer, 1.5 mM or 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 U *Taq*. DNA polymerase, 0.25 μ M of each primer (18, 21), and 2 μ L of the boiled sample in a final volume of 25 μ L. Each reaction was overlaid with sterile mineral oil and subjected to 25 cycles of denaturation at 92°C for 1min, annealing at 62°C for 1 min and extension at 72°C for 2 min after an initial denaturation at 95°C for 5 min. Amplification products were completed by a final extension step of 72°C for 10 min. The PCR products were electrophoresed in 1% agarose gels in 10 μ L quantities (16) and visualized after staining with ethidium bromide by uv transillumination. The sequences of oligonucleotide primers used in this study are listed in Table 1 (18).

Table 1. Sequences of oligonucleotide primers used in this study (18)

Primer	Nucleotide sequence(5-3)	16S rDNA position ^a
DIV1F	CGC ACT GGT TAA TACCTG GTG	455- 475
DIV1R	CTA CCG TGG TAA TCG CCC TCC	1454-1474
DIV2F	CGC TTC GGT TAA TAC CTG GAG	455-475
DIV2R	CTG CCG TGG TAA TCG CCC CCC	1454-1474

^a*Escherichia coli* numbering system (23)

RESULTS AND DISCUSSION

Crude preparations from infected potato and tomato plants were tested by PCR with the specific primers DIV1F / DIV1R and DIV2F / DIV2R. The PCR tests with the primer pair DIV2F / DIV2R successfully detected *R. solanacearum* in all experimentally and naturally infected samples either with or without visible symptoms (Fig. 1, 2). The extract from symptomatic and symptomless tubers and stems of potato plants inoculated with biovar 2 isolates gave a characteristic 1019 bp band with primer pair DIV2F / DIV2R. Specific primers detected the bacterium in potato plants grown at 23 or 28°C showing wilt and vascular deterioration. All potato plants were dead at 28°C after 14 days. At 18°C some potato plants remained symptomless while others showed epinasty, but all gave a PCR product with DIV2F / DIV2R primer pair. Infection of plants with *R. solanacearum* biovar 2 was identified in the mother and daughter tubers, and stems of potato plants by PCR and DIV2F / DIV2R primer pair. Tomato plants showed symptoms of epinasty and wilt at 23°C and 28°C one week after inoculation with biovar 2 isolates. The extracts from tomato-infected plants were tested with the specific primers. All infected tomato plants at 23°C and 28°C gave a PCR product with specific DIV2F / DIV2R primer pair. At 18°C, however, they showed neither symptoms nor any PCR products (Fig. 3). The PCR tests with the primer pair DIV1F + DIV1R (specific for biovars 3, 4 and 5) did not amplify DNA in any infected potato and tomato samples with or without symptoms as expected (Fig. 4). None of the healthy control potato and tomato plants gave any visible PCR products with specific primers.



Fig. 1. Electrophoretic analysis of PCR products with DIV2F + DIV2R of *Ralstonia solanacearum*. Lane 1: standard molecular marker, (100bp DNA Ladder, Sinagen) Lane

2: ACH 158, Lane 3-10: infected potato from field, Lane 11: healthy potato tuber, Lane 12: water control

Rapid, sensitive and specific detection methods are required to identify latent bacterial wilt infection in planting materials, especially for quarantine and certification purposes. The PCR tests with specific DIV2F / DIV2R primers permitted successful identification of latent infection of potato and tomato plants. The results indicated that the application of the PCR with specific primers enabled the detection of infection of potato and tomato

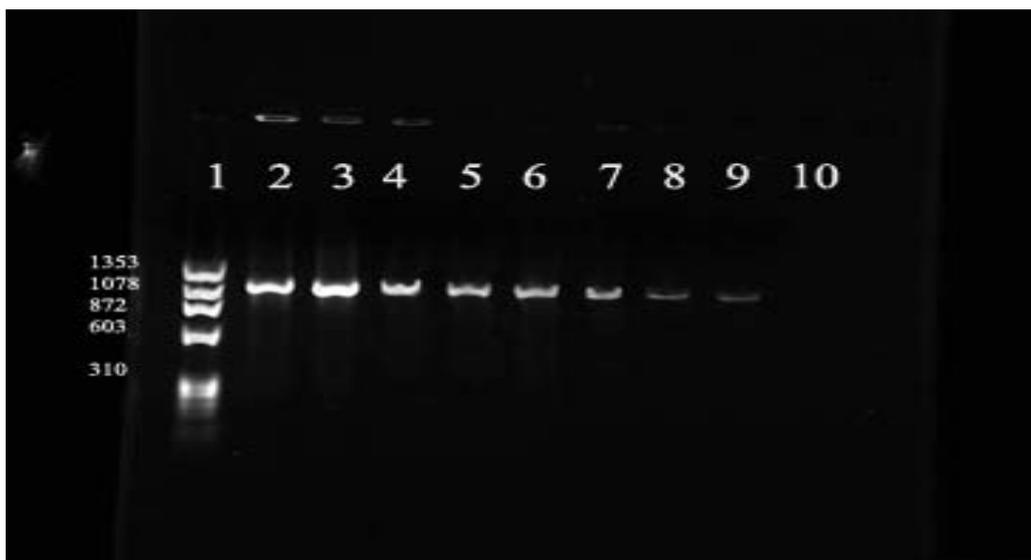


Fig. 2. Electrophoretic analysis of PCR Products with DIV2F + DIV2R of *Ralstonia solanacearum*. Lane 1: standard molecular marker, (100bp DNA Ladder, Sinagen) Lane 2: ACH 158, Lane 3-6: infected potato with symptoms, Lane 7-9: infected potato without symptoms, Lane 10: water control

plants without prior isolation and enrichment of pathogen from plant tissues. Potato daughter tubers produced at an 18°C growing condition did not exhibit any deterioration of vascular tissues, but they generated a visible PCR product with specific primers. The PCR test was not positive with primer pair DIV1F / DIV1R specific for biovars 3, 4 and 5 with all infected potato and tomato samples with or without visible symptoms. There is no report of existence of biovar 3, 4 and 5 of *R. solanacearum* in Iran (2, 3). The sensitivity of these primers was sufficient for the detection of *R. solanacearum* isolates in latently infected plants (18). There are many reports of the spread of bacterial wilt of potato and banana within and between countries in latently infected planting materials (1, 7, 8). At low temperatures, potato plants are latently infected and symptom expression does not occur (14). *R. solanacearum* biovar 2 (race 3) isolates are low temperature adapted and have a close association with potato (9). The wide distribution of *R. solanacearum* biovar 2 isolates in cool temperate regions of the world is probably due to close association with potato plants, including debris and latently infected tubers, and the transport of seed tubers over long distances (9). Seed potato tubers and many weeds have been shown to be symptomless carriers of *R. solanacearum* and there are probably also

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many unknown hosts that maintain high levels of inoculums between successive crops (11).

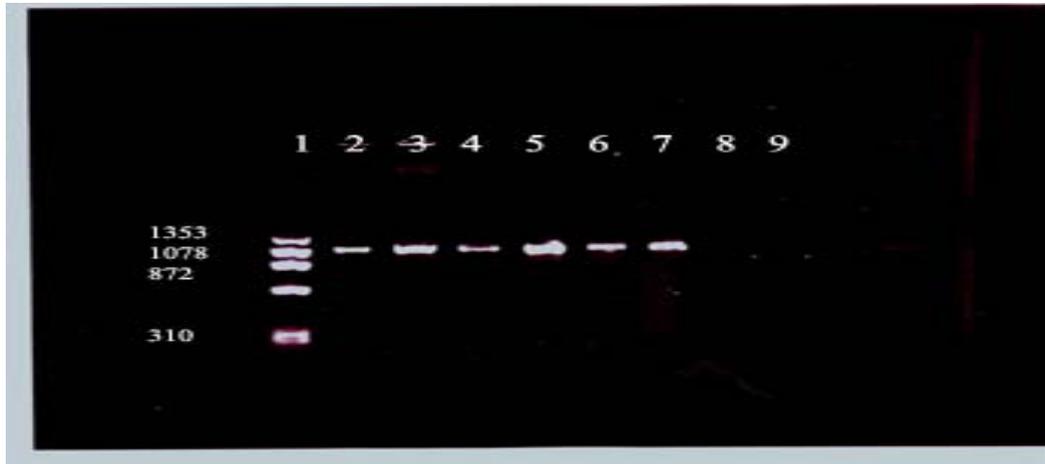


Fig. 3. Electrophoretic analysis of PCR products with DIV2F + DIV2R primers of *Ralstonia solanacearum*.

Lane 1: standard molecular marker (100bp DNA Ladder, Sinagen)

Lane 2, 3, 4: tomato inoculated with biovar 2 isolate and incubated at 23°C,

Lane 5, 6: tomato inoculated with biovar 2 isolate and incubated at 28°C,

Lane 7: ACH 158 (Positive control)

Lane 8: healthy tomato

Lane 9: water control

The application of the PCR with specific primers enables detection of latent infection. The PCR assay combines speed, sensitivity and specificity which are critical parameters for any detection assay. Results from the comparison of ELISA and PCR tests for the

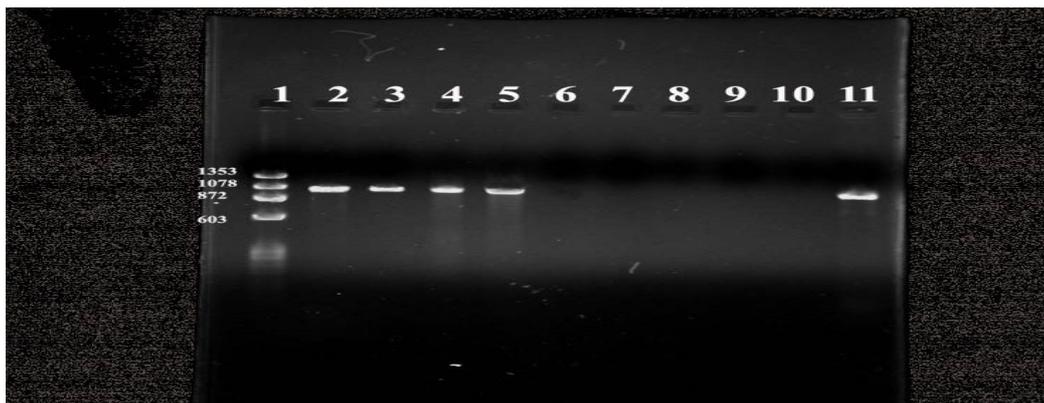


Fig. 4. Electrophoretic analysis of PCR products with DIVIF+DIV1R and DIV2F+DIV2R primers of *Ralstonia solanacearum*

Lane 1: standard molecular marker (100bp DNA Ladder, Sinagen)

Lane 2, 3, 4, 5: daughter tubers inoculated with biovar 2 isolates and amplified by DIV2F + DIV2R Primers

Lane 6, 7, 8, 9: daughter tuber inoculated with biovar 2 isolates and amplified by DIV1F + DIV1R Primers

Lane 10: water control

Lane 11: ACH 158, positive control with DIV2F + DIV2R

detection of *R. solanacearum* in seed tubers, under laboratory conditions, showed that the PCR technique is 1000-10000-fold more sensitive than ELISA (19, 20). One of the limitations of PCR, however, is the inhibition of DNA polymerase by phenolics and oxidases present in many plants (17). These inhibitory compounds cause false negatives or low detection sensitivities in PCR tests. The combination of serology and PCR technology (immunocapture PCR), is an alternative and less expensive technique which can be used to remove inhibitory compounds from plant extracts or soil (17).

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