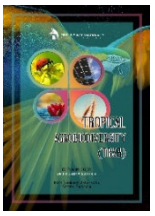
ZIBELINE INTERNATIONAL™ PUBLISHING
ISSN: 2716-7046 (Online)
CODEN: TARGCA

Tropical Agrobiodiversity (TRAB)

DOI: http://doi.org/10.26480/trab.02.2024.106.109



RESEARCH ARTICLE

PCR BASED DETECTION OF LATENTLY INFECTED POTATO WITH BROWN ROT PATHOGEN, RALSTONIA SOLANACEARUM

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ARTICLE DETAILS

Article History:

Received 24 September 2024
Revised 28 October 2024
Accepted 02 October 2024
Available online 25 November 2024

ABSTRACT

Brown rot of potato is caused by a soil borne bacterium, *Ralstonia solanacearum*(Smith), a major limiting factor in the production of many crop plants around the world. An experiment was conducted to assess the status of brown rot disease in potatoes with the development of a rapid detection technique and approaches for production of brown rot free export quality potatoes to boost up potato export from Bangladesh. Survey results revealed a variation in latent infection (%) in tuber samples collected from six major growing areas viz. Dinajpur, Rangpur, Bogra, Joypurhat, Munshigonj and Comilla. The lowest (30%) latent infection was detected in tuber samples collected from Joypurhat as compared to Rangpur and Munshigonj in case of Asterix. However, in Diamant, the lowest (40%) latent infection was observed in Rangpur while 80% latent infection was recorded in Dinajpur and 88% was recorded in Munshigonj. Intriguingly, no latent infection was detected in Cardinal collected from Dinajpur while 40% latent infection was detected in samples from Joypurhat. However, 60% latent infection was detected in Granola which was collected from Dinajpur. The detection of *R. solanacearum* was reported in latently infected tubers by PCR using primers corresponding to 16SrDNA. In this study, PCR with primers PS-1 and PS-2 (16S rDNA) was used to detect *R. solanacearum* in latently infected tubers. An amplicon size corresponding to 553bp were obtained using enriched tuber extract as template.

KEYWORDS

PCR Detection, Brown rot pathogen, Management, Export quality, Potatoes

1. INTRODUCTION

Brown rot of potato caused by the Gram-negative bacterium Ralstonia solanacearum (Smith) is considered as a great threat for potato production in tropical and subtropical regions of the world including Bangladesh (Yabuuchi et al., 1995; Janse, 1988; Hayward, 1991). The quarantine importance of brown rot pathogen increased in temperate Europe following several outbreaks in Sweden, Belgium, England, France, Germany, Italy, Netherlands, Portugal and Spain, North and Latin America with zero tolerance (Olsson, 1976; MuÈller, 1996; Elphinstone, 1996; Stead, 1996). The vascular pathogen *R. solanacearum* causes wilting of potato plants and rotting of tubers; however, it also survives latently in potato tubers without causing symptoms (Ciampi et al., 1981). Transmission of the brown rot bacterium to disease-free regions may be attributed to the movement of latently infected seed potatoes, where the organism remains viable and pathogenic and then causes disease under favourable conditions after planting, or to the irrigation of potatoes with contaminated surface water (Janse, 1988; Olsson, 1976; Elphinstone, 1996; Anonymous, 1997). Since the pathogen is mainly transmitted through tuber seed, the use of healthy planting materials is the most effective means to control the disease (Hayward, 1991). The major components for brown rot free potato production are the detection of latent infection of brown rot pathogen, *R. solanacearum* in seed potato as well as the planting potato in *R. solanacearum* free soil. However, detection of *R. solanacearum* in soil by streaking soil solution onto a specific medium is limited due to its low sensitivity (Granada and Sequeira, 1983). Therefore, the effective management of brown rot disease is largely based on the detection of latent infection of *R. solanacearum* in seed potato. In Europe, a number of standard methods were developed to monitor the occurrence of *R. solanacearum* in potato seed tubers which includes enzyme-linked immunesorbent assay (ELISA), the bioassay on tomato plants, detection by *R. solanacearum*-specific DNA sequences by polymerase chain reaction (PCR) using enriched potato extract sample (OEPP/EPPO, 1990, Priou et al., 1998, Pastrik and Maiss, 2000 and Priou et al., 2001). However, there is no method reported for the detection of brown rot pathogen in seed potato in our country which is now mandatory for our quarantine and certification schemes in order to produce brown rot free potato. Potato exports have witnessed a sharp rise by 56% worth to US$ 19m in the current fiscal which promote the export of the food item and also to save the farmers by ensuring fair prices to their product. Bangladesh exports potato to different countries including Russia, other European countries and Malaysia, the largest importer of potato from Bangladesh. However, brown rot disease caused by the bacterium *R. solanacearum* has been detected recently in potato exported from Bangladesh by Russia. Consequently, Russian Government has decided to ban the import of potato from Bangladesh. Therefore, development of

Quick Response Code	Access this article online	
	Website: www.trab.org.my	**DOI:** 10.26480/trab.02.2024.106.109

Cite the Article: Md. Mokarram Hossain, Md. Rashidul Islam, Ronzon Chandra Das, Md. Mahbubul Haque, Mohammad Delwar Hossain and Faria-Ter Jerin (2024). PCR Based Detection of Latently Infected Potato with Brown Rot Pathogen, Ralstonia Solanacearum. *Tropical Agrobiodiversity, 5(2): 106-109*.

efficient detection techniques of brown rot pathogen, *R. solanacearum*is essential for routine use in quarantine procedures and seed certification schemes. Knowledge obtained from this enhances our understanding to formulate efficient management strategies in producing brown rot free export quality potatoes.

2. Materials and Method

2.1 Survey and Potato Sample Collection

Six commercially potato growing areas viz. Dinjapur, Rangpur, Bogra, Joypurhat, Munshigonj and Comilla were selected for brown rot assessment. Six hundred tubers/seed lot were selected randomly and collected from heaps at harvest or from stores within 4 weeks of harvest for each variety. Three fields were selected in each area and a total of fifteen fields were selected exhibiting the incidence of bacterial wilt (BW) covering an area of 0.25 to 1.5 ha under each area. BW incidence was scored according to the number of wilted plants observed among a total of 400 plants per hectare by examining 20 rows of 20 plants following a cross-pattern design.

2.2 Sample Testing

Tubers were randomly divided into 24 composite samples of 25 tubers each. For the detection of *R. solanacearum,* tubers were processed as described by (Priou et al., 2001). After tuber disinfection with 10% clorox, thin slices or strips (3 x 3 mm) of the tuber were removed with a scalpel from around the stolon end along the vascular ring. The tuber fragments were put into a plastic bag on ice and weighed. Two ml of sterile citrate buffer (0.1 M citric acid, 0.1 M sodium citrate, pH 5.6) per g of tuber tissue were added, and tuber fragments were squashed with wooden roll or a rubber mallet. The enriched tuber extracts were prepared by mixing 500 µl of the supernatant tuber extract with 500 µl of M-SMSA in a 1.5 ml Eppendorf tube, and incubating for 48 h at 30°C with constant agitation (170 rpm) or manual agitation twice a day (Elphinstone et al., 1996).

2.3 Preparation of DNA Template

One ml of enriched tuber extract was centrifuged at 12,000 rpm for 5 min followed by washing the pellet for three times with nuclease-free water for each sample. It was then resuspend in 20 µl of nuclease-free water and boiled for 15 min to denature DNA. Samples were then being kept at -20 ºC until use.

2.4 Primers, PCR Conditions and Visualization of the Amplicon

PS-1(Forward)(5'-AGTCGAACGGCAGCGGGGG-3') and PS-2 (Reverse) (5'-GGGGATTTCACATCGGTCTTGCA-3') primer pair was used for the detection of *R. solanacearum* in the enriched tuber extract. PCR reactions were performed in 25 µl of reaction mix with 0.01 U/ µl of *Taq* DNA polymerase (Takara, Japan). Amplifications were performed with a Thermo Cycler with PCR conditions as initial denaturation at 95^{0}c for 5 min, followed by 35 reaction cycles of 95^{0}c for 30s, 68^{0}c for 30s and 72^{0}c for 45s (Pastrik and Maiss,2000). After the final reaction cycle, the mixture was kept at 72^{0}c for 5 min and stored at 4^{0}c. A 553 bp fragment were visualized after staining with 0.5 pg m1^{-1} ethidium bromide by loading a 15 µl of the PCR products.

3. Results and Discussion

The results obtained from this study on the development of a PCR based rapid detection technique for brown rot pathogen *Ralstonia solanacearum* in latently infected potato has been presented in this portion.

3.1 Variation in Latent Infection of Potato Tubers

Variation in latent infection (%) in potato tubers was observed among the districts surveyed. In case of Asterix, lowest (30%) latent infection was detected in tuber samples collected from Joypurhut as compared to Rangpur and Munshigonj with similar (60%) infection. In case of Diamant, lowest (40%) latent infection was observed in Rangpur while 80% latent infection was recorded in Dinajpur and 88% was recorded in Munshigonj. In case of Cardinal, no latent infection was detected in samples collected from Dinajpur while 40% latent infection was detected in Joypurhat (Figure 1A, 1B and 1C). However, 60% latent infection was detected in potato tuber samples (cv. Granola) collected from Dinajpur. This variation in latent infection might be due to the variation in host resistance responses and pathotypic variation of *R. solanacearum*. However, these factors need to be addressed by identifying resistance gene expression in potato varieties and analyzing the genetic variation of *R. solanacearum* population in different growing regions. It was reported that the genetic variation of *R. solanacearum* isolates causing bacterial wilt of brinjal and potato obtained from different growing areas of Bangladesh (Nishat et al., 2015).

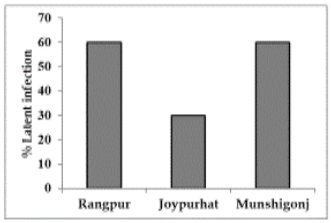

Fig.1A

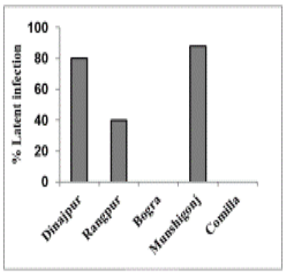

Fig.1B

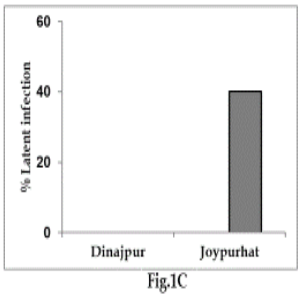

Fig.1C

Figure 1A, 1B, 1C: Showing infection status of Potato varieties of Asterix, Diamant and Cardinal respectively.

3.2 Detection of *Ralstonia Solanacearum* in Latently Infected Potato Tubers

3.2.1 Morphological detection of Ralstoniasolanacearum

A loop full of the enriched tuber extract was striked to a culture medium containing TTC. *R. solanacearum* was identified based on the colony morphology (red centre and whitish margin) from some samples. It was found that the colonies appeared with red centre and whitish margin were termed as virulent. *R. solanacearum* was previously detected on TTC plates based on morphology by a number of researchers in home and abroad (Rahman et al. 2011; Ahmed et al. 2013; Nishat et al. 2015; Kelman,1954). Again, Granada and Sequeira in 1983 described the detection of *R. solanacearum* morphologically (Granada and Sequeira, 1983). French et al., used TTC culture medium for isolation, identification and maintenance of *R. solanacearum* (French et al., 1995). They all found TTC medium to be very useful to identify and isolate *R. solanacearum.*

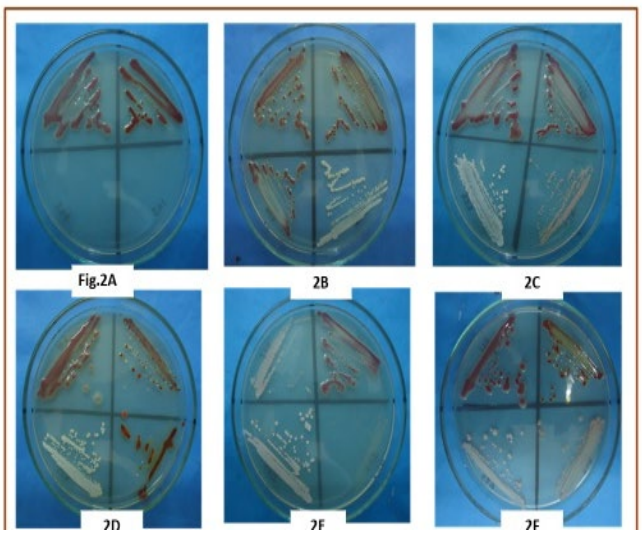

Figure 2: Colonies of *R. solanacearum* on TTC medium. Detection was done by streaking a loopfull of enriched tuber extract on TTC plates. Plates were photographed after 48 hours of incubation at 28^{o}C.

3.3 PCR Based Detection of *Ralstonia Solanacearum* from Enriched Tuber Extracts and Genomic DNA

In Europe, a number of standard methods were developed to monitor the occurrence of *R. solanacearum* in potato seed tubers which included enzyme-linked immunosorbent assay (ELISA), the bioassay on tomato plants, detection by *R. solanacearum*-specific DNA sequences by

polymerase chain reaction (PCR) using enriched potato extract sample (OEPP/EPPO, 1990; Priou et al., 1998; Pastrik and Maiss, 2000 and Priou et al., 2001). Again, a study reported PCR based detection of *R. solanacearum* in potato tubers using the primers PS-1/PS-2 and OLI I/Y2 (Seal et al., 1993).

PCR products obtained from the enriched tuber extracts went for running in agarose gel and gel documentation (Elphinstone et al., 1996). A Primer set of PS-1, PS-2 was used and an amplicon size of 553 bp corresponding to 16S rRNA region confirmed the presence of *R.solanacearum* in the enriched tuber extracts. *R. solanacearum* in the positive samples were also identified on TTC plates (figure 3A).

Genomic DNA was obtained from morphologically identified *R. solanacearum* using Wizard(R) Genomic DNA Purification kit. Then the DNA templates went for PCR with two sets of primers naming PS-1, PS-2 and Y-2, OL-1.

In case of Primers PS-1and PS-2, an amplicon size 553 bp corresponding to 16S rRNA region confirmed the presence of *R. solanacearum* in the positive samples as identified on TTC plates (figure 3B). Research reported the PCR based detection of *R. solanacearum* in potato tubers using the primers PS-1/PS-2 (Pastrik and Maiss, 2000; Priou et al., 2001; Seal et al., 1993). In case of Y-2 and OL-1 Primers, an amplicon size of 288 bp corresponding to 16S rRNA region confirmed the presence of *R. solanacearum* in the positive samples as identified on TTC plates (figure 3C) and indicated the latent infection of potato tubers with *R. solanacearum.* Many scientists all over the world have used this primer for PCR based specific detection of the brown rot pathogen. (Ashmawy, 2015) identified some isolates of *R. solanacearum*at the molecular level through Polymerase Chain Reaction (PCR) using a pair of specific primers (OLI-1 and Y2) that produced a 288 bp specific PCR product.

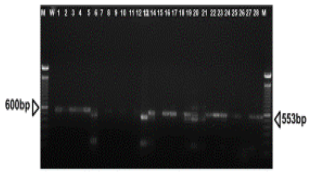

Fig.3A Detection of R. solanacearum from the enriched tuber extracts of some representative positive samples (samples from which R. solanacearum detected on TTC medium) by PCR using primers PS-1 and PS-2.

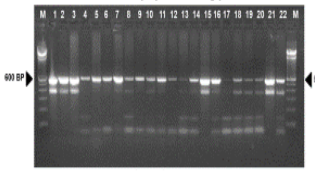

Fig.3B Detection of R. solanacearum in latently infected potato tubers from the extractracted genomic DNA of some representative positive samples (samples from which R. solanacearumdetected on TTC medium) by PCR using primers PS-1 and PS-2.

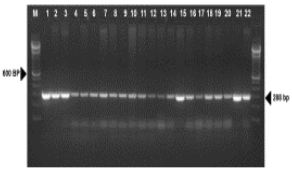

Fig.3C Detection of R. solanacearum in latently infected potato tubers from the extracted genomic DNA of some representative positive samples (samples from which R. solanacearum detected on TTC medium) by PCR using primers Y-2 and OL-

Figure 3: Detection of *R. solanacearum* in latently potato tubers from the extracted genomic DNA of some representative positive samples.

3.4 Status of Brown Rot of Potato in Major Growing Areas

Variation in latent infection (%) in potato tubers was observed among the districts surveyed.In case of Asterix, lowest (30%) latent infection was detected intuber samples collected from Joypurhut as compared to Rangpur and Munshigonj with similar (60%) infection. In case of Diamant, lowest (40%) latent infection was observed in Rangpur while 80% latent infection was recorded in Dinajpur and 88% was recorded in Munshigonj. In case of Cardinal, no latent infection was detected in samples collected from Dinajpur while 40% latent infection was detected in Joypurhat (figure 1A, 1B and 1C). However, 60% latent infection was detected in potato tuber samples (cv. Granola) collected from Dinajpur. This variation in latent infection might be due to the variation in host resistance responses and pathotypes variation of *R. solanacearum*. However, these factors need to be addressed by identifying resistance gene expression in potato varieties and analyzing the genetic variation of *R. solanacearum* population in different growing regions. A study reported the genetic variation of *R. solanacearum* isolates causing bacterial wilt of brinjal and potato obtained from different growing areas of Bangladesh (Nishat et al., 2015).

3.5 Detection of Brown Rot Pathogen, *R. Solanacearum* on TTC Medium

R. solanacearum was detected in the enriched tuber extract by streaking a loopful of enriched culture in medium containing tetrazolium chloride (TZC). *R. solanacearum* was identified based on the colony morphology (red centre and whitish margin) from some samples as shown (figure 2A, 2C and 2D) while *R. solanacearum* were not detected in some samples (figure 2B, 2E and 2F*). R. solanacearum* were previously detected on TTC plates based on morphology by a number of researchers in home and abroad (Rahman et al. 2011; Ahmed et al. 2013; Nishat et al. 2015; Kelman,1954).

3.6 PCR Based Detection Technique for Brown Rot Pathogen, *R. Solanacearum*

The performances of different treatments were assessed in terms of per cent latent infection of tubers. The latent infection in potato tubers were detected by PCR using enriched tuber extract with PS-1 and PS-2 primers as described earlier by (Pastrik and Maiss 2000).An amplicon size 553 bp corresponding to 16SrRNA region confirmed *R. solanacearum* in the positive samples as identified on TTC plates (figure 3). In Europe, a number of standard methods were developed to monitor the occurrence of *R. solanacearum* in potato seed tubers which includes enzyme-linked immunosorbent assay (ELISA), the bioassay on tomato plants, detection by *R. solanacearum*-specific DNA sequences by polymerase chain reaction (PCR) using enriched potato extract sample (OEPP/EPPO, 1990;Priou et al. 1998; Pastrik and Maiss, 2000 and Priou et al. 2001).

4. Conclusion

A variation in latent infection in tuber samples collected from six major growing areas viz. Dinajpur, Rangpur, Bogra, Joypurhat, Munshigonj and Comilla were recorded and latent infection varied from variety to variety and region to region.*R. solanacearum* was detected in latently infected tubers by PCR using specific primers PS-1 and PS-2 corresponding to 16SrDNA with an amplicon size corresponding to 553bp.

Acknowledgement

This research work was financed by The Ministry of Science and Technology, Govt. of the People's Republic of Bangladesh. Md. Mokarram Hossain (Student id: 15 P.Path JD 01M, Reg. No: 36318) was awarded with National Science and Technology Fellowship/2015 from The Ministry of Science and Technology, Govt. of the People's Republic of Bangladesh, for the research.

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