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PCR-BASED SPECIFIC DETECTION OF *RALSTONIA SOLANACEARUM* IN SOIL, SEED AND INFECTED PLANT MATERIAL

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ABSTRACT

DNA-based methods have provided powerful tools to identify and detect microorganisms with high sensitivity and specificity. PCR assay amplifies the DNA of bacterial pathogens, targeting the species-specific sequences in their genome. In the present study an efficient DNA isolation protocol and PCR based detection of bacterial wilt pathogen in soil, seed and infected plant materials has been described. The specific primers 759f/760r was successfully used to detect *Ralstonia solanacearum* from different sources and predicted 281-bp DNA fragment was obtained. In conclusion, the PCR-based detection method using *R. solanacearum* specific primer offers a rapid and sensitive method for unambiguous detection of this pathogen in soil, seed and infected tomato plant materials.

KEYWORDS: **Bacterial wilt, *Ralstonia solanacearum*, DNA, PCR**

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INTRODUCTION

Bacterial wilt of tomato (*Solanum lycopersicum* Mill.) is a major disease inhibiting tomato production in tropical and subtropical regions of the world. The symptoms of the disease can be recognized by sudden wilting of the plant during hot and sunny days, which results from blockage of the plant's water conducting vessels. The disease is caused by the soil-borne bacterium *Ralstonia solanacearum* [1]. Controlling tomato bacterial wilt 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. Moreover, it can colonize host plants like members of the Solanaceae and non-host plants including many weeds, without producing visible symptoms. These carriers serve as a primary inoculum source for the subsequent tomato crop. *R. solanacearum* can persist at low populations in naturally infested soil for many years without a host plant [2] and the population size could increase to the plant infection threshold within a season after the host plants are returned to the fields. 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.

Several detection methods have been developed for *R. solanacearum.* 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. These traditional methods of detection and identification are often time consuming and laborious. In order to optimize the efficiency of prophylactic measures, powerful tools for the identification and detection of the bacterium in diverse substrates (plant, seeds and soil) are required. DNA-based methods have provided powerful tools to identify and detect microorganisms with high sensitivity and specificity. DNA amplification of pathogen offers many advantages over classical techniques; neither purification nor cultivation of the pathogen is required and the specificity, sensitivity and response time of tests are improved. PCR assay amplifies the DNA of bacterial pathogens, targeting the species-specific sequences in their genome. 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 [3, 4]. Until now there is a very few validated PCR protocol recommended for the detection of *R. solanacearum* in samples especially in soil samples. Therefore, this study was to describe standard protocols for reliable detection of *R. solanacearum* whatever the origin of the sample may be. Recently, PCR techniques have been applied to identify *R. solanacearum* strains using specific primers [5, 6].

The significant advantage of PCR is potential for detecting very small numbers of bacteria in the environment, especially in soils. The objective of this work was to describe the more sensitive method for the extraction of DNA and amplification by PCR using specific primers for *R. solanacearum* from soil, seeds and infected tomato plant materials, which would be suitable for use in routine laboratory testing programmes.

MATERIALS AND METHODS

(i) Bacterial culture, soil, seed samples and infected plant material

Field surveys were conducted in major tomato growing districts of Karnataka, India [7]. Soil samples, seed samples and infected plant material were collected from 31 different tomato fields during field survey. Field survey, Isolation of *R. solanacearum* and maintenance on Kelman's TZC [8] medium in detail as given in our previous publication [7]. Briefly, the suspected plant material, soil samples and fruits were collected from the field survey, brought to the laboratory and isolated the target pathogen from the diseased plants. Collected plant materials

were surface sterilization with 70% ethyl alcohol followed by three repeated washings with distilled water and blot-dried. Then the plant parts (0.5–1 cm) were plated onto the petriplates containing Kelman's TZC agar medium. Plates were incubated at 28 ± 2 ^{0}C for 24–48 h. The virulent (white fluidal irregular colonies with pink centre) colonies of *R. solanacearum* were subcultured from the inoculated plates.

(ii) DNA extraction from bacterial culture

Ralstonia solanacearum isolates were sub cultured onto Kelman's TZC medium and incubated at 28 ± 30 ^{o}C for 48 h. A loopful of culture was inoculated into conical flask containing 10 ml of nutrient broth and incubated overnight with shaking (160 rpm) at 30 ^{o}C. 1.5 ml aliquots were drawn from the 10 ml culture, centrifuged at 13,000 rpm for 3 min and the supernatant was poured off. 200 µl of lysis buffer was added to the pellet and mixed vigorously with the pipette. 66 µl of 5 M NaCl was added and centrifuged at 13,000 rpm for 10 min. Then the supernatant was transferred to a new tube and 1 µl of RNase A (10 mg /ml) was added to that, mixed well and incubated at 37 ^{o}C in a water-bath for 30 min. Equal volume of chloroform / isoamyl alcohol (24:1) was added, mixed gently by inverting tubes and centrifuged at 13,000 rpm for 6 min. With a micropipette, the upper aqueous phase was carefully transferred to a clean tube. 2.5 or 3-fold volume of cold 95 % ethanol was added, mixed well but gently and the tubes were left in the freezer (-20 ^{o}C) for 30 min to 1 h and then centrifuged at 13,000 rpm for 6 min. Ethanol was gently poured off carefully and washed with 2-fold volume of 70 % ethanol, centrifuged at 13,000 rpm for 10 min. Ethanol was gently poured off carefully and air-dried. The pellet was resuspended in 50 µl of 1x TE, left overnight at 4 ^{o}C and DNA was stored at -20 ^{o}C.

(iii)DNA extraction from soil

DNA was extracted from soil as described by [9] with a slight modification in the present study. Each soil sample (1 g) was mixed with 2.7 ml of DNA extraction buffer (100 mM TrisHCl (pH 8), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% Cetyl Trimethyl Ammonium Bromide (CTAB), 20 µl of proteinase K (10 mg/ml) and 300 µl of 20 % sodium dodecyl sulfate (pH 8.0) in a mortar and then ground with a pestle. The sample was transferred into a centrifugation tube and incubated in a 65 ºC water bath for 2 h with gentle end-over-end inversions every 20 min. The supernatant was collected after centrifugation at 6000 x *g* for 10 min, transferred into a new tube and then mixed with an equal volume of chloroform-isoamyl alcohol (24:1 v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 vol of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids was obtained by centrifugation at 15 000 x *g* for 20 min at room temperature, washed with 80 % ethanol, and resuspended in 100 µl of TE (10 mM TrisHCl, 1 mM EDTA, pH 8.0).

(iv) DNA extraction from seed samples and infected plant material

One g of tomato seeds and plant materials collected during field survey were crushed separately in liquid nitrogen using pestle and mortar and mixed with 2.7 ml of DNA extraction buffer (100 mM TrisHCl (pH 8), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1 % Cetyl Trimethyl Ammonium Bromide (CTAB), 20 µl of proteinase K (10 mg/ml) and 300 µl of 20 % sodium dodecyl sulfate (pH 8.0) in a mortar and then ground with a pestle. The sample was transferred into a centrifugation tube and incubated in a 65 ºC water bath for 2 h with gentle end-over-end inversions every 20 min. The supernatant was collected after centrifugation at 6000 x *g* for 10 min, transferred into a new tube and then mixed with an equal volume of chloroform-isoamyl alcohol (24:1 v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 vol of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids was obtained by centrifugation at 15 000 x *g* for 20 min at room temperature, washed with 80% ethanol, and resuspended in 100 µl of TE (10 mM TrisHCl, 1 mM EDTA, pH 8.0).

(v) Molecular detection of R. solanacearum by PCR amplification and DNA sequencing

For specific detection of *R. solanacearum* the specific primers 759f (5'-GTCGCCGTCAACTCACTTTCC-3') & 760r (5'-GTCGCCGTCAGCAATG CGGAATCG-3') were selected according to [6] and custom synthesized from Bangalore Genei, Bangalore, India. The specific primers 759f and 760r corresponds to the upstream region of *lpxC* gene of *R. solanacearum*, used to identify *R. solanacearum* at the species level and amplify a predicted 281-bp DNA fragment. DNA isolated from *R. solanacearum* bacterial culture, soil, seed samples and infected plant material were used as a template for PCR amplification of upstream region of *lpxC* gene. DNA was amplified in 25 µl of reaction mixture containing 1x PCR buffer, 2.5 mM of each dNTP, 10 pmole/µl of primers, 3U/µl of Taq DNA polymerase, 100 ng/µl of genomic template DNA. The PCR tubes were given a small spin, and were placed in a PCR thermocycler (Eppendorf, Germany) and programmed as follows: The thermal cycle consisted of initial denaturation at 94 ºC for 3 min, annealing at 53 ºC for 1 min, and extension at 72 ºC for 1.5 min, followed by 30 cycles of 94 ºC for 15 s, 60 ºC for 15 s, 72 ºC for 15 s, and a final extension at 72 ºC for 5 min. PCR products were gel electrophoresed in 1.2 % Agarose-gel-Electrophoresis and visualized under UV light. The 281-bp DNA fragment was then excised from the agarose gel, then purified using GeneJET™ Gel Extraction kit, Fermentas and sent for sequencing at Chromous Biotech, Bangalore, India. Then the obtained sequence was BLAST searched and multalined with the NCBI database and submitted to NCBI Gene Bank.

RESULTS

(i) PCR amplification for the detection of R. solanacearum using specific primers

The specific primers used for the detection of the isolated *R. solanacearum,* amplified an expected band of 281-bp as reported by [6]. Total 9 suspected isolates showed the 281-bp amplification with the specific primers. This indicates the isolated bacterial cultures were *R. solanacearum* culture (Fig. 1).

(ii) PCR amplification for the detection of R. solanacearum in soil using specific primers

Extraction of DNA from soil was successful for the detection of *R. solanacearum* by PCR assay. The quality and quantity of DNA obtained by using DNA extraction method from soil can be used for PCR analysis. Following the direct DNA extraction from each soil sample, a 1 µl aliquot of crude DNA was used as a template for PCR reaction, resulting in an amplification of 281-bp fragment by *R. solanacearum* specific 759f and 760r primers (Fig. 2). Total 18 different soil samples collected during the field survey showed the amplification with the specific primers.

(iii) PCR amplification for the detection of R. solanacearum in seed samples and infected plant material

The quality and quantity of DNA obtained by using DNA extraction method from seed samples and infected plant materials can be used for PCR analysis. Under UV, single band of 281-bp DNA fragment was amplified from all the infected seed samples and infected plant materials with 1.2 % agarose gel. This indicated the presence of *R. solanacearum* in both seed samples and in the infected plant materials (Fig. 3).

(iv) DNA sequencing

The Sequencing data obtained from the Chromous Biotech revealed 232 bp. The NCBI Blast search and multalinment revealed 100 % homology with the *lpxC* upstream region of *R. solanacearum* databases in the NCBI genebank (Accession No EU348769.1, EU348768.1 and EU348767.1,). Then the obtained sequence was deposited to the NCBI genebank with the accession No. GQ379236 (Fig 4. and Fig. 5).

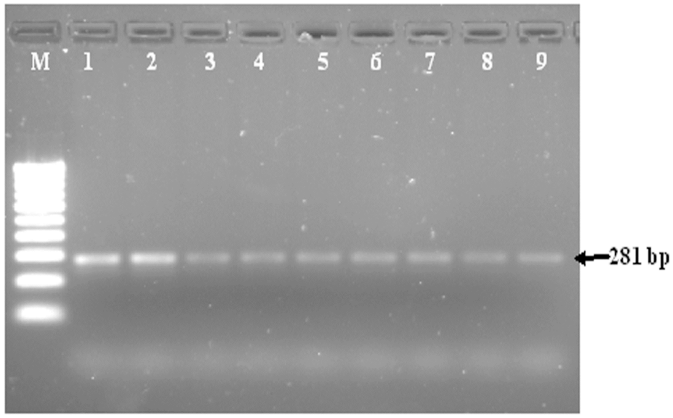

Figure 1

Polymerase Chain Reaction amplifications of 281-bp fragments (shown with an arrow) from different isolates of R. solanacearum from different parts of Karnataka using specific primers. M: 100-bp DNA marker, Lane 1-9: DNA extracted from different isolates of R. solanacearum.

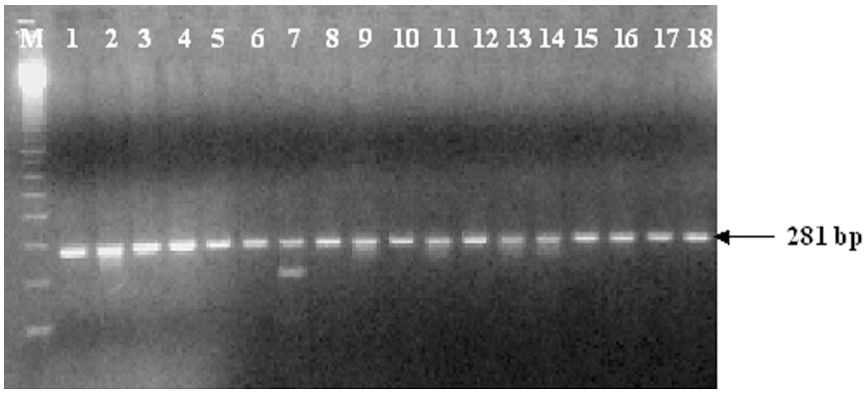

Figure 2

Polymerase Chain Reaction amplifications of 281-bp fragments (shown with an arrow) from different soil samples collected from different parts of Karnataka using specific primers. M: 100-bp DNA marker, Lane 1-18: DNA extracted from different soil samples.

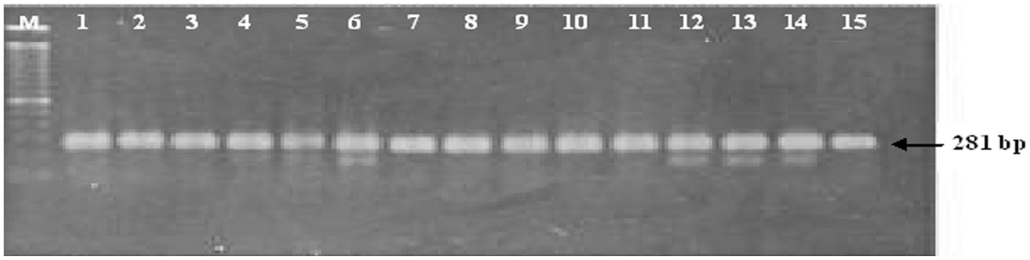

Figure 3

Polymerase Chain Reaction amplifications of 281-bp fragments (shown with an arrow) from different seed samples using specific primers. M: 100-bp DNA marker, Lane 1-8: DNA extracted from different seed samples, Lane 9-15: DNA extracted from infected plant material.

5'CGGTTGGGATAACCCGAAGCATACCATTTTCACGTGCCTGCCTCCAAAACGACTTTTGTATCG
GGATGTTAGGCCCTGCCATAGTCTGCTCACGATTTCAGCACGCAGCCACCATGCCGTTGCGTCA
TCGGCCGCACGGCGGTGCGGGATGCAGGACAGGACTCGGCCGTTCGGAGGGGTGCGGGTGG
CGTCAGAGGCGCGCCAGCATCGATTCCGCATTGCTGACGGCGAA-3'

Figure 4
Partial genomic sequence of R. solanacearum lpxC upstream region (Accession No. GQ379236).

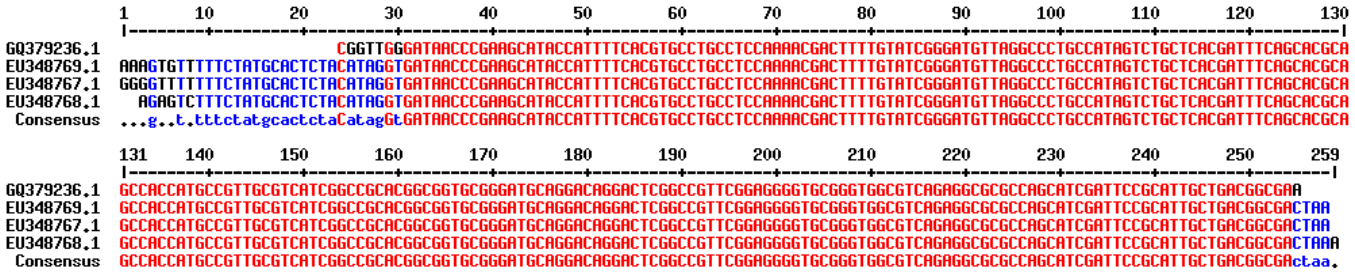

Figure 5
Sequence alignment of R. solanacearum lpxC upstream region (Acc. No. Q379236) with NCBI database (Acc. No EU348769.1, EU348768.1 and EU348767.1) showing 100 % homology.

DISCUSSION

DNA based methods have provided powerful tools to identify and detect microorganisms with high sensitivity and specificity. In the present study, *R. solanacearum* were detected based on molecular techniques. Recent advances in the field of molecular genetics have led to new possibilities for pathogen detection that depend on the recognition of DNA sequences that are specific only to the pathogen genome or to that of a particular strain of the pathogen that is to be differentially detected. DNA amplification by PCR is a highly sensitive and less laborious method to detect pathogen-specific DNA. PCR has become a useful tool for detection of plant pathogenic bacteria including *R. solanacearum.* DNA detection techniques have an obvious advantage over cultural methods in that they do not require bacteriological expertise to differentiate the pathogen from the saprophytic bacterial contaminants of the preparation. Furthermore, the DNA-based methods can detect and identify the pathogen in a much shorter length of time than is required to culture and purify the organism and then perform large numbers of laborious physiological and biochemical tests to confirm its identification. Specific primers have been developed earlier for detection of *R. solanacearum* strains using PCR. Primer pair 759f and 760r were developed by the[6] for the detection of *R. solanacearum* which amplify a 281-bp fragment from all *R. solanacearum* strains.

This study made use of the 281-bp DNA fragment that can be amplified through PCR using the primer pair 759f and 760r. Previous reports showed that this 281-bp band could be amplified from *R. solanacearum* strain from Australia, the Phillippines, Taiwan, Japan and America[6,10].Present study revealed that the 281-bp fragment was conserved also among the Indian *R. solanacearum* isolates suggesting that the primers 759f/760r have potent for specific detection of this bacterium. Many earlier reports documented the results similar to our results. The phylogenetic relationships of *R. solanacearum*, BDB and *P. syzygii* were determined based on the 282-bp sequences of the PCR amplified fragment obtained using specific primers 759 and 760 [11].The *R. solanacearum* specific primers (759f and 760r) for PCR amplification were used to confirm the presence of *R. solanacearum* in ginger plants [12].

Soil is vehicle for the spread of *R. solanacearum* and a high level of wilting occurred in soils previously planted with susceptible plant cultivars [1].For effective quarantine measures and disease control, it is important to develop a specific, rapid and sensitive detection method for *R. solanacearum* in soil. The application of

PCR in soil analyses is limited by the labor-intensive extraction of total DNA from soil.In this study we have extracted the DNA directly from soil and used for PCR assay using *R. solanacearum* specific primer. Similarly the viable cells of *R. solanacearum* in soil were detected using PCR techniques with specific primers predicting the 281-bp band [10]. The specific primers were designed for the detection of *R. solanacearum* in soil samples by PCR [13]. The detection assay was developed to detect *R. solanacearum* in soil and weeds by combining immunocapture and the polymerase chain reaction (IC-PCR) [3]. An efficient DNA isolation protocol and PCR based detection of bacterial pathogen in soil was described [14]. In the present study DNA was isolated directly from the seed samples, infected plant materials and used as template for PCR assay using specific primers.The 281-bp band confirmed the infection of *R. solanacearum* in all the seed samples and infected plant materials. The PCR assay for the detection of *R. solanacearum* in potato tubers and leaves were developed [15, 16]. The bacterial endophytes were also identified using PCR technique by amplifying their 16S rDNA region [17]. In conclusion, PCR is a reliable, sensitive and specific detection technique for the detection of *R. solanacearum* from soil, seed samples and also from the infected plant materials. The use of this PCR and the simple DNA extraction method from soil, seed samples and infected plant materials offer a rapid method for the identification and detection of *R. solanacearum.* The specific primers used in this study were shown to be specific to *R. solanacearum.*

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REFERENCES

1. Hayward AC, Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum.* Ann Rev Phytopathol, 2: 65 – 87, (1991).
2. Akiew EB, Influence of soil moisture and temperature on the persistence of *Pseudomonas solanacearum*. In: Persley GJ (eds), *Bacterial wilt disease in Asia and the South Pacific.* Proceedings of an international workshop held at PCARRD, Los Banos, Philppines, 8-10 October, 1986, pp. 77 – 79. ACIAR Proceedings No. 13.
3. Dittapongpitch V, and Surat S, Detection of *Ralstonia solanacearum* in soil and weeds from commercial tomato fields using immunocapture and the polymerase chain reaction. J Phytopathol, 151: 239 – 246, (2003).
4. Schaad NW, Jones JB, and Chun W, Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd Edn, APS Press, St. Paul, Minnesota, USA pp 373. (2001).
5. Seal SE, Jackson LA, and Daniels MJ, Isolation of a *Pseudomonas solanacearum* specific DNA probe by subtraction hybridization and construction of a species-specific oligonucleotide primers for sensitive detection by polymerase chain reaction. Appl Environ Microbiol, 58: 3751 – 3758, (1992).
6. Opina N, Tavner F, Holloway G, Wang J, Li T, Maghirang R, Fegan M, Hayward A, Krishnapillai V, Hong W, Holloway B, and Timmis J, A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). Asia Pac J Mol Biol Biotech, 5 (1): 19 – 30, (1997).
7. Vanitha SC, Niranjana SR, Mortensen CN, and Umesha S, Bacterial wilt of tomato in Karnataka and its management by

Pseudomonas fluorescens. BioControl, 54: 685 – 695, (2009).
8. Kelman A, The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on tetrazolium medium. Phytopathology, 64: 693 – 695, (1954).
9. Zhou J, Bruns MA, and Tiedje JM, DNA recovery from soils of diverse composition. Appl Environ Microbiol, 62: 316 – 322, (1996).
10. Ito S, Ushijima Y, Fujii T, Tanaka S, Kameya-Iwaki M, Yoshiwara S, and Kishi F, Detection of viable cells *Ralstonia solanacearum* in soil using a semiselective medium and a PCR technique. J Phytopathol, 146: 379 – 84, (1998).
11. Villa J, Tsuchiya K, Horita M, Natural M, Opina N, and Hyakumachi M, DNA analysis of *Ralstonia solanacearum* and related bacteria based on 282-bp PCR-amplified fragment. Plant Dis, 87: 337 – 1343, (2003).
12. Kumar A, Methods for screening ginger (*Zingiber officinale* Rosc.) for bacterial wilt resistance. Ind Phytopathol, 59 (3): 281 – 286, (2006).
13. Lee YA, and Wang CC, The design of specific primers for the detection of *Ralstonia solanacearum* in soil samples by polymerase chain reaction. Bot Bull Acad Sin, 41: 121 – 128, (2000).
14. Kumar A, and Anandaraj M, Method for isolation of soil DNA and PCR based detection of ginger wilt pathogen, *Ralstonia solanacearum*. Ind Phytopathol, 59 (2): 154 – 160, (2006).
15. Pastrik KH, and Maiss E, Detection of *Ralstonia solanacearum* in potato tubers by polymerase chain reaction. J Phytopathol, 148: 619 – 626, (2000).
16. Ibrahim MA, Metry EA, Moustafa SA, Tawfik AE, and Nasr El-Din TM, Morphological and molecular diagnosis of commercial potato cultivars infected with *Ralstonia solanacearum.* Arab J Biotech, 8 (2): 211 – 222, (2005).
17. Deljou A, Mousaviehzade M, Ghasemi A, and Rahimian H, Identification of *Mycobacterium* sp. as an alfalfa root bacterial endophytes using 16s rRNA gene sequence analysis. International Journal of Pharma and Bio Sciences, 1 (2): 1– 8, (2010).