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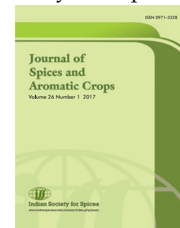

Detecting and monitoring endophytic colonization by *Pseudomonas putida* BP25 in black pepper (*Piper nigrum* L.) using quantitative real-time PCR

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Abstract

A quantitative real-time PCR assay was developed to quantify *Pseudomonas putida* BP25, an antagonistic endophyte against a broad range of pathogens in black pepper such as *Phytophthora capsici, Colletotrichum gloeosporioides*, *Rhizoctonia solani, Gibberella moniliformis, Athelia rolfsii* and a plant parasitic nematode, *Radopholus similis*. The real-time PCR primers were designed based on the16S rRNA sequences of *P. putida* strains and specificity of the primers was confirmed. The detection limit of the assay was found to be 1 pg. The assay detected and quantified the bacterial colonization in the roots at weekly intervals after inoculation. The *P. putida* DNA was quantified to be 0.4 ng in roots corresponding to 5.4 log_{10} CFU g^{-1} at 7th and 14th day after inoculation (DAI). A decline in endophyte population was observed during 21st and 28th DAI and the DNA concentration ranged from 3.7-4.6 pg corresponding to 3.4-3.5 log_{10} CFU g^{-1} of root. No amplification could be obtained in stem and leaf samples. The newly developed real-time PCR could be useful for detection, quantification and monitoring of endophytic *P. putida* BP25 in different plant tissues.

Keywords: black pepper, endophytic colonization, *Pseudomonas putida*, real- time PCR

Introduction

Endophytic bacteria are known to promote plant growth by direct plant growth promotion and by protecting plants against diseases. The control of plant diseases is achieved either by direct microbial antagonism or by inducing resistance in plants (Compant *et al.* 2010). *Pseudomonas putida* BP25 is a Gram negative endophytic bacterium, initially isolated from the internal tissues of black pepper roots after surface sterilization (Aravind *et al*. 2009). Foot rot caused by *Phytophthora capsici* and slow decline caused by *Radopholus similis* are the major production constraints in black pepper (*Piper nigrum* L.) and *P. putida* BP25 was found to suppress *P. capsici* lesions and *R. similis* population in black pepper (Aravind *et al*. 2009 & 2010). *P. putida* BP25 was also reported to produce microbial volatile organic compounds (MVOCs) which inhibited a broad range of pathogens such as *P. capsici, Colletotrichum gloeosporioides, Rhizoctonia solani, Gibberella moniliformis, Athelia rolfsii* and a plant parasitic nematode *R. similis* (Sheoran *et al.* 2015). Bacterial colonization is a prerequisite for exerting their biocontrol or growth promotion

effects in plants and there are several techniques to investigate the colonization. Generally, identification and estimation of bacterial population in plant tissues is done by classical and molecular techniques (Hallmann *et al.* 1997). In black pepper, rhizoplane and endogenous population of *P. putida* BP25 was estimated by dilution plating with the aid of antibiotic resistance and confirmed by Bio-PCR using *tpiA* primer (Sheoran *et al*. 2015). In order to estimate bacterial population, culturing methods have been widely used. But it is laborious and time consuming. Though Bio-PCR is the most sensitive method which could detect very low populations of bacteria, it is not suitable for quantitative analysis as it involves culturing of the bacteria before amplification.

Real-time PCR offers the opportunity to quantify bacteria without the need of tagging the strains before plant inoculation and is reported to be highly sensitive (Boeckman *et al*. 2000). Real-time PCR can amplify and simultaneously quantify a target DNA without any post amplification analysis, and it is introduced for quantification of bacteria in plant tissues (Ruppel *et al*. 2006). It has been successfully used for quantification of endophytic fungi (Macia-Vicente *et al*. 2009; Landa *et al*. 2013), pathogenic bacteria (Oliveira *et al*. 2002; Gervasi & Scortichini 2009) and endophytic bacteria (Lacava *et al.* 2006; Pereira *et al.* 2014; Sorte *et al*. 2014) in plant tissues. Primers coding for 16S rRNA sequences have been widely used for quantifying bacteria using real-time PCR (Lacava *et al.* 2006; Gervasi & Scortichini 2009; Timmusk *et al.* 2009; Sorte *et al*. 2014). The objective of the present study was to develop a reliable and sensitive real-time PCR method for detecting and quantifying *P. putida* BP25 in tissues of black pepper pre-colonized with the bacterium.

Materials and methods

Bacterial strains

Pseudomonas putida BP25 was initially isolated from the internal tissues of black pepper root after surface sterilization (Aravind *et al*. 2009). The spontaneous rifampicin resistant strain tagged with *gfp* designated as BP25R::gfp was obtained from IARI, New Delhi and used in this study. The bacterium was routinely grown on Luria Bertani Agar (LBA, Himedia) with rifampicin (50 μg mL^{-1}) and gentamicin (75 μg mL^{-1}) at 28°C for 24 h. For checking the specificity of primers, a bacterium from the same genera -*Pseudomonas aeruginosa* IISR853 and other endophytic bacterial strains such as *Bacillus megaterium* BP17 and *Curtobacterium luteum* TC10 were used. These bacteria were grown on Tryptone soya agar (TSA, Himedia) at 28°C for 24 h.

Plant inoculation

Black pepper (*Piper nigrum* L.) variety Sreekara was used in this study. The cuttings were grown on sterilized soil and maintained at greenhouse conditions at 26-30°C with 60-80% humidity. For inoculating the cuttings, BP25R::gfp cell suspension O.D- 1.0, corresponding to approximately 10^8 CFU mL^{-1}, was prepared. The two-leaf stage cuttings were inoculated by dipping the roots in the cell suspension and then planted in sterilized soilrite. Plants inoculated with sterile distilled water served as control and were maintained under same conditions.

Quantitative real-time PCR

Design of P. putida *specific primers*

The primers were designed based on the 16S rRNA sequences of *P. putida* strains deposited in the NCBI GenBank. An extensive BLAST search was performed and 16S rRNA gene sequences of *P. putida* strains such as D15, S16, BD18-S16, AGL14, RNAB, RW10S2, KBL2RM2, R20 and R43 were used. These 16S rRNA gene sequences were aligned using BioEdit sequence alignment editor (Ibis Bioscience, USA). Within these sequences unique regions specific to the organism were identified and primers were designed using Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers were synthesized by IDT (USA). In this study, primer sequences 16S SS1_FP: 5'-CAACGTTTCGAAAGGAACG-3'

and 16S SS1_RP: 5'-GTCGCCTTGGTGAGCCATT-3' yielding a 131 bp amplicon were used.

Specificity and sensitivity of the assay

In order to check the specificity of the primers, real-time PCR assays were done with DNA of different bacterial species (Pereira *et al.* 2014). The bacterial DNA preparations were made from pure cultures grown in Tryptone soya broth (TSB, Himedia) for 24 h. The broth culture was centrifuged at 14,000 rpm for 2 min at room temperature. The supernatant was discarded and pellet was washed with sterile distilled water three times. DNA was extracted according to Kumar *et al*. (2004). Bacterial cells were lysed in N-cetyl N, N, N-trimethyl ammonium bromide (CTAB) extraction buffer. The DNA was extracted using standard chloroform/isoamyl alcohol extraction protocol and precipitated using isopropanol. The quantity and quality of the DNA were determined using Bio-Photometer (Eppendorf, Germany) and it was stored at -20°C. Real-time PCR reactions were carried out in a final volume of 25 μL reaction mixture containing 12. 5 μL QuantiFast SYBR green mastermix (Qiagen, Germany), 1 μL template containing 10 ng DNA and 0.5 μM forward and reverse primers in a Rotor-Gene Q Real-time PCR system (Qiagen, Germany). Thermal cycling conditions were as follows: an initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 10 sec, annealing at 50°C for 15 sec and extension at 60°C for 20 sec.

Performance of the assay was analyzed by running 2.5 fold-serial dilutions of *P. putida* BP25R::gfp DNA. Different DNA concentrations ranging from 26 ng to 1 pg per reaction were run using the same real-time PCR parameters described above. The BP25R::gfp cell suspension, used for DNA extraction, was also serially diluted and number of cells (CFU mL^{-1}) was calculated. The real-time PCR runs were analyzed using automatic software settings. Cycle threshold (C_T) values obtained were plotted against serial dilutions of template DNA and a standard curve was constructed. The amplification efficiency was determined using the equation $E= (10^{(-1/s)}-1) \times 100$, where *s* is the slope obtained from standard curve.

Quantification of P. putida *BP25 in black pepper*

The bacterized and non-bacterized plants were sampled at different intervals; 7th day, 14th day, 21st day and 28th day. Three plants per time point were taken. The plants were partitioned into roots, stems and leaves and surface sterilized with 0.5% sodium hypochlorite for 20 min followed by 70% alcohol for 1 min and rinsed in sterile distilled water thrice. Surface sterilized plant parts (100 mg) were used for total DNA extraction using CTAB buffer (Ausubel *et al*. 2002). The DNA was extracted using phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated using isopropanol. The extracted DNA was quantified using Bio-Photometer (Eppendorf, Germany) and used for real-time PCR reactions. All the samples were tested using the same parameters described above.

Results and discussion

Specificity and sensitivity of the assay

Primer specificity was assessed using genomic DNA isolated from *P. putida* BP25R::gfp, another *Pseudomonas* sp., *P. aeruginosa* IISR853 and other bacterial genera such as *B. megaterium* BP17 and *C. luteum* TC10. Primers were able to detect only BP25R::gfp DNA by real-time PCR confirming its specificity (Fig. 1). No non specific amplification could be detected with other tested bacteria. The sensitivity of the primers was determined using serial dilutions of genomic DNA isolated from BP25R::gfp ranging in the concentration from 26 ng to 1 pg. Adequate correlations of C_T versus DNA quantity were obtained. C_T values ranged from 16.4 to 30.7. The real-time PCR assay could detect DNA at a concentration as low as 1 pg (Fig. 2a). The C_T values were plotted relative to the corresponding serial dilution of the template DNA and a standard curve was plotted (Fig. 2b). The reaction parameters of the real-time PCR assay such as slope, amplification efficiency and correlation coefficient were determined from the standard curve. The standard curve

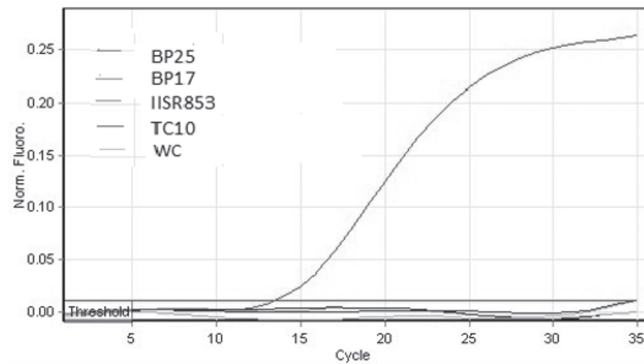

Fig. 1. Real-time PCR to assess the specificity of the primers; amplification is shown with only *Pseudomonas putida* BP25::gfp DNA confirming its specificity.

a

Norm. Fluoro.
Threshold
Cycle

b

$y=-3.361x+20.998$
$R^2=0.99$

CT
Concentration

Fig. 2. a) Amplification plot showing adequate amplification corresponding to different concentrations of DNA; b) Real-time PCR standard curve constructed by plotting threshold cycle (C_T) against different serial dilutions of *Pseudomonas putida* BP25R::gfp DNA.

presented a slope value of -3.36, amplification efficiency 98% and correlation coefficient R^2=0.99 indicating a good fitness of the real-time assay.

Quantification of P. putida *DNA in black pepper*

The DNA extracted from different plant parts of black pepper rooted cuttings inoculated with BP25R::gfp cell suspension was used as a template for real-time PCR. DNA extracted from non-bacterized plants was also run simultaneously. The real-time PCR assay was able to detect *P. putida* DNA in roots at all the different intervals; 7th day, 14th day, 21st day and 28th day. C_T values ranged from 21.8 to 22.6 at 7th and 14th days after inoculation and, 28.2 to 29.8 at 21st and 28th days after inoculation (Fig. 3). C_T values obtained were interpolated in the standard curve to calculate the BP25R::gfp DNA concentration with respect to the total. The DNA concentration was determined to be 0.4 ng at 7th day and 14th day. DNA concentrations at 21st and 28th day were lower and were found to be 3.7 and 4.6 pg, respectively. The DNA concentrations were correlated to the number of bacterial colonies (CFU mL^{-1}) obtained in corresponding serial dilutions. Number of bacterial colonies was determined to be 5.4 log_{10} CFU g^{-1} of root at 7th and 14th day after inoculation and, 3.4-3.5 log_{10} CFU g^{-1} of root at 21st and 28th day after inoculation. No amplification could be detected in stem and leaf samples and with non-bacterized plant parts. Negative controls also showed no amplification signal.

The biocontrol effects of *P. putida* through direct antagonism and induced resistance against pathogens were proved in different plant species (Chen *et al.* 1995; Adam *et al*. 2008; Validov *et al*. 2009). An endophytic *P. putida* BP25, a promising biocontrol agent was isolated from black pepper root tissues (Aravind *et al.* 2009). Since then, its beneficial effects such as production of volatile organic compounds and induction of defense responses have been revealed (Sheoran *et al*. 2015 & 2016). Effective colonization of root along with the interior parts of plants was demonstrated to be an important trait for biocontrol activity and culture dependent techniques were generally employed for colonization studies (Quadt-Hallmann *et al*. 1997; Berg & Hallmann 2006). Culture independent techniques are gaining importance for rapid and sensitive analyses of bacterial population quantification. Real-time PCR has been developed as an invaluable tool for estimating the population levels of endophytes and quantification of *Herbaspirillum seropedicae* strain SmR1, an endophytic diazotroph in maize roots (Pereira *et al.* 2014) and *Gluconacetobacter diazotrophicus* in sugarcane

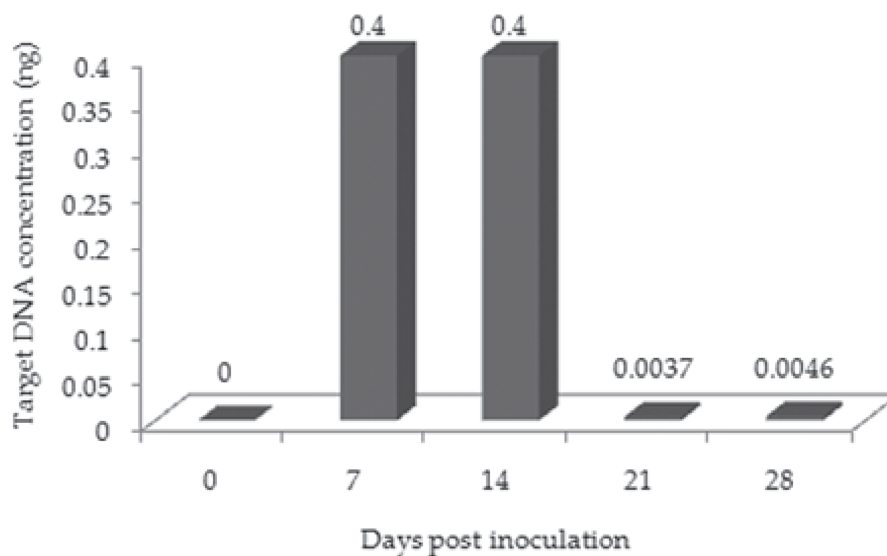

Fig. 3. Real-time PCR quantification of *Pseudomonas putida* BP25R::gfp DNA in black pepper roots

by real-time PCR were reported (Sorte *et al.* 2014).

A real-time PCR method was developed to quantify the endogenous population of artificially inoculated *P. putida* BP25 in black pepper. In our study, the detection limit was found to be 1 pg which is equivalent to 62 cells (1.8 log_{10} CFU mL^{-1}). Previously, detection limit of 1 pg was observed with *Pseudomonas syringae* pv. *aesculi* in horse chestnut (Green *et al.* 2009) and *Paenibacillus polymyxa* in wild barley (Timmusk *et al.* 2009). The endogenous bacterial population could be quantified using total DNA extracted from black pepper roots and a population of 5.4 log_{10} CFU g^{-1} of root was obtained at 7th and 14th day and 3.4-3.5 log_{10} CFU g^{-1} of root at 21st and 28th day. Similarly, a population density of 4-5 log_{10} CFU g^{-1} was reported with *Gluconacetobacter diazotrophicus* in sugarcane (Sorte *et al*. 2014) and *Methylobacterium mesophilicum* in *Catharanthus roseus* (Lacava *et al.* 2006). In our earlier study, the colony count was reported to be 2.8 to 3.4 log_{10} CFU g^{-1} of root at 7th and 14th day and, 1.15 log_{10} CFU g^{-1} of root at 21st and 28th day after inoculation using dilution plating (Sheoran *et al*. 2015). In this study, the bacterial cell numbers measured using real-time PCR were two orders of magnitude higher than the populations reported using dilution plating. This may be because of the inability of real-time PCR to differentiate between dead and live cells (Pathak *et al.* 2012). However, no amplification could be obtained in the uninoculated plants showing the specificity of the assay. In conclusion, the present study presents a rapid, reliable and sensitive real-time PCR assay for detection and quantification of *P. putida* BP25 in black pepper.

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