Sensitive and specific detection of *Xanthomonas hortorum* pv. *pelargonii* in geranium by real-time PCR

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Abstract: *Xanthomonas hortorum* pv. *pelargonii* is the causal agent of bacterial blight of geranium. A specific and rapid real-time PCR assay for detecting the bacterium in plant was developed in this study. We compared sensitivity of conventional and real-time PCR for detection of the pathogen in inoculated plants. For application to disease management programs, PCR amplification must be able to detect latent infections of asymptomatic geraniums. Our results displayed that conventional PCR lacks sufficient sensitivity to be used as diagnostic tools for detection of *X. hortorum* pv. *pelargonii* in latent infections. On the other hand, real-time PCR is suitable method for detection of latent infection of the bacterium in planting materials and can be considered in management programs. The ability for accurate and reliable detection of *X. hortorum* pv. *pelargonii* in asymptomatic tissue is a significant step in setting up "pathogen free" certification programs for bacterial blight of geranium.

Key words: bacterial blight, conventional PCR, detection, geranium, real-time PCR

Introduction

Pelargonium species are affected by several pathogens like *Botrytis cinerea*, *Puccinia pelargonii-zonalis*, *Pythium* sp., *Rhodococcus fascians* and *Ralstonia solanacearum* (Nameth *et al*. 1999). Bacterial blight caused by *Xanthomonas hortorum* pv. *pelargonii* (formerly called *X. campestris* pv. *pelargonii*) is the most destructive disease of geraniums and its occurrence results in unmarketable plants and major economic losses. Symptoms consist of water soaked spots and V-shaped lesions on leaves, wilting of the plant and black lesions on stems (Daughtrey and Macksel 1993; Nameth *et al*. 1999). Plants may be infected with the bacteria and not show symptoms when grown under conditions of low temperature and humidity (Daughtrey and Wick 1993). Cuttings from latently infected plants used for propagation are the major means of dispersal the bacterium (Daughtrey and Wick 1993). The use of disease-free planting material is the best practical technique for management the disease. Therefore, sensitive and accurate detection of *X. hortorum* pv. *pelargonii* is vital to timely elimination of infected plant and vegetative propagation. Diagnosis of diseases of geranium was previously performed by different methods. Firstly, plant materials were incubated in nutrient broth to detect the existence of particular fungi and bacteria. This technique was called "culture indexing". If the broth became cloudy with bacterial or fungal growth, parent plant was discarded (Oglevee-O'Donovan 1986). Clearly, this manner was not able to accurate identification. Then, diagnosis of bacterial blight of geranium was based on the combination of isolation and serological technique. In this method, identification of the disease was carried out by polyclonal antibodies (Anderson and Nameth 1990; Tuinier and Stephens 1989). False-negative result due to variation in the surface antigens of the pathogen was the major disadvantage of this technique. Thus, latent infections often were not distinguished. Additionally, fatty acid analysis was used to identify *Xanthomonas*. This procedure had ability to identify *X. hortorum* pv. *pelargonii* isolates (Sasser 1990; Hodge *et al*. 1992). Because fatty acid analysis require special facilities and expertise, this method was not appropriate for identifying the pathogens. Manulis *et al*. (1994) used random amplification polymorphism DNA (RAPD) to identify pathovar *pelargonii*. Using a single 1.2 kb region as a pathovar-specific fragment, they designed specific primers for diagnosis the bacterium. Moreover, Sulzinski *et al*. (1996) planned other specific primers to detect the bacterium in infected geraniums. Sulzinski *et al*. (1998) used a procedure including biological enrichment and polymerase chain reaction (PCR) amplification to detect small populations of *X. hortorum* pv. *pelargonii* in tissues of geranium. Immediately after inoculation, sections of the petioles and stems were harvested and incubated for 24 or 48 h in nutrient broth. After total DNA extraction, PCR amplification with specific primers was used to detect the pathogen (Sulzinski *et al*. 1998).

To date, no real-time PCR assays have been developed specifically for detection of *X. hortorum* pv. *pelargonii* in geranium. The objective of this research was to compare sensitivity of conventional and real-time PCR for detection of *X. hortorum* pv. *pelargonii* in florist's geranium (*Pelargonium* × *hortorum*). In the present study, we showed

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that real-time PCR has ability to detect latent infections of bacterial blight of geranium.

Materials and Methods

Primer design for real-time PCR

Primers XhqF (5′-CGCTACCAAAAGGCAAAGAG) and XhqR (5′-GCACTGGCAACCTCATATTC) were designed in the present work using AlleleIDH version 6 software for detection of *X. hortorum* pv. *pelargonii* by real-time PCR with a predicted PCR product of 125 bp. This pair primer was designed based on an unique 197 bp sequence in *X. hortorum* pv. *pelargonii* that previously described by Sulzinski *et al*. (1998). To determine their potential annealing specificity, the primer sequences based on the unique 197 bp sequence were tested by similarity search against the sequence databases in National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi/).

Bacterial strains, culture conditions and DNA extraction

Bacterial strains used in this study are listed in Table 1. Bacterial strains were cultured on nutrient agar (peptone – 0.5%, NaCl – 0.5%, yeast extract – 0.2%, beef extract – 0.1%, agar – 1.5%) at 28°C for 3 days. Genomic DNA from the bacterial strains was extracted using Genomic DNA isolation Kit (Denazist, Iran) according to manufacturer's instruction. The quantity and purity of genomic DNA was measured using NanoDrop spectrophotometer. Serial dilution of extracted DNA (ranging from 2 µg to 200 fg) was prepared to use as template for conventional and real-time PCR.

Conventional and SYBR Green real-time PCR

Conventional PCR was conducted using primers XcpM1 (5′-ACGCGCTACCAAAAGGCAAAGAG) and XcpM2 (5′-GATCTGCGGTTGTCCTGAAGATTGG) that previously described by Sulzinski *et al*. (1996). The PCR reactions were carried out in a total volume of 25 µl containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 200 µM each of dNTPs, 100 ng of primers, 1 unit of Taq polymerase and 1 µl of DNA template. The PCR was performed in a Palm-cycle™ (Life Science, Germany). The thermal cycling program was 95°C for 5 min, 30 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 10 min. A 10 µl aliquot of the reactions was checked by agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light. The SYBR Green real-time PCR assay was performed in the real-time PCR system (Bioneer, South Korea) with the following program: 95°C for 5 min, 40 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 10 min. After the final amplification cycle, a melting-curve analysis was performed to prove specific amplification. Each experiment was conducted in three repetitions. To confirm the specificity of the primers for *X. hortorum* pv. *pelargonii*, the template DNAs extracted from control bacterial strains (Table 1) were used in real-time PCR assay.

Table 1. Bacterial strains used in specificity tests of real-time PCR assay

No.	Species	Host//Habitat	Geographical origin	Result
1	*Xanthomonas hortorum* pv. *pelargonii*	*Pelargonium* × *hortorum*	Iran	+
2	*X. hortorum* pv. *pelargonii*	*Pelargonium* × *hortorum*	Iran	+
3	*X. hortorum* pv. *pelargonii*	*Pelargonium* × *hortorum*	Iran	+
4	*X. hortorum* pv. *pelargonii*	*Pelargonium* × *hortorum*	Iran	+
5	*X. hortorum* pv. *pelargonii*	*Pelargonium* × *hortorum*	Iran	+
6	*X. hortorum* pv. *pelargonii*	*Pelargonium* × *hortorum*	Iran	+
7	*X. hortorum* pv. *pelargonii*	*Pelargonium* × *hortorum*	Netherlands	+
8	*X. hortorum* pv. *pelargonii* (type strain)	*Pelargonium* × *hortorum*	UK	+
9	*X. citri* subsp. *citri*	*Citrus sinensis*	Iran	–
10	*X. axonopodis* pv. *phaseoli*	*Vigna radiata*	Iran	–
11	*X. arboriacola* pv. *juglandis*	*Juglans regia*	Iran	–
12	*X. euvesicatoria*	*Capsicum annuum*	Iran	–
13	*Rhodococcus fascians*	*Pelargonium* × *hortorum*	Iran	–
14	*Pseudomonas syringae* pv. *syringae*	*Armeniaca vulgaris*	Iran	–
15	*Pseudomonas syringae* pv. *lachrymans*	*Cucumis sativus*	Iran	–
16	*Agrobacterium tumefaciens*	*Vitis sylvestris*	Iran	–
17	*Streptomyces scabies*	*Solanum tuberosum*	Iran	–
18	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*	*Vigna radiata*	Iran	–
19	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	Iran	–
20	*Ralstonia solanacearum*	*Solanum tuberosum*	Iran	–
21	*Pseudomonas fluorescens*	soil	Iran	–
22	*Bacillus* sp.	soil	Iran	–

In planta detection of *Xanthomonas hortorum* pv. *pelargonii*

Xanthomonas hortorum pv. *pelargonii* was grown on nutrient agar plate and bacterial cells were adjusted to an optical density at 600 nm (OD_{600}) = 1. Then, 40 µl of the suspension injected into stems of plants. Plants were incubated at 28°C with 60% relative humidity (RH). The inoculated plants were monitored during a 10-day period for disease incidence (the number of plants showing symptoms divide by the number of total plants). Total DNA extraction from stems was performed using genomic DNA isolation kit (Denazist, Iran) following the manufacturer's protocol. Conventional and real-time PCR were carried out as described above. The *in planta* assay was performed in triplicate for each sample.

Results

Specificity of designed primers

The specificity of primers XhqF and XhqR based on an unique 197 bp sequence in *X. hortorum* pv. *pelargonii* was tested by similarity search against the NCBI BLAST sequence database (http://www.ncbi.nlm.nih.gov/). When comparing the sequence of the 125 bp product amplified by the primer pair, there was no significant match with sequences of other bacterial strains. Moreover, no fluorescent detection was disclosed in any of control bacterial samples in real-time PCR amplifications with the primers XhqF and XhqR, which indicated that the pair of primers was highly specific for detection of *X. hortorum* pv. *pelargonii* (Table 1).

Sensitivity test by conventional and real-time PCR

Conventional PCR was able to detect *X. hortorum* pv. *pelargonii* in DNA samples ranging from 2 µg to 20 ng of extracted DNA. Lower amounts of DNA were undetectable by conventional PCR (Fig. 1). In comparison with conventional PCR, real-time PCR was more sensitive for detection of the bacterium (Table 2; Fig. 2). This procedure detected the bacterium even in the lowest amount of extracted DNA (200 fg).

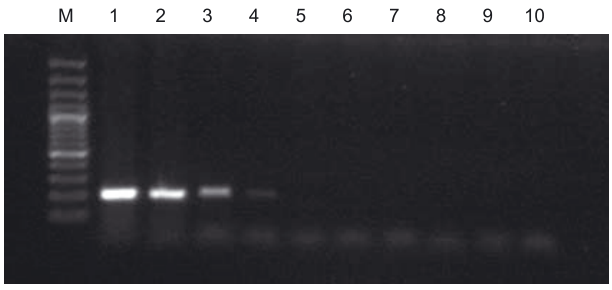

Fig. 1. Ethidium bromide-stained PCR amplicons (197 bp) following amplification of the DNA templates extracted from *X. hortorum* pv. *pelargonii* with primers XcpM1/Xcpm2 (Sulzinski *et al*. 1996): Lane M – DNA molecular marker; Lane 1 – positive control; Lane 2 to 9 – ranging from 2 µg to 200 fg of extracted DNA from the bacterium; Lane 10 – negative control

In planta detection of the pathogen

The first *in planta* detection by real-time PCR was positive 4 days after inoculation, whereas the first plants showing symptoms were observed 5 days after inoculation. Real-time PCR was more sensitive in detection of *X. hortorum* pv. *pelargonii* in the samples collected 4–7 days after inoculation compared to conventional PCR. Moreover, when all inoculated plants displayed symptoms, conventional and real-time PCR were able to detect *X. hortorum* pv. *pelargonii* with 100% efficiency (Table 3).

Table 2. Ct-values related to amounts of extracted DNA from *Xanthomonas hortorum* pv. *pelargonii* by real-time PCR

Amounts of DNA	Ct (mean±SE)
Positive control (3.4 µg)	11.43±0.11
2 µg	12.72±0.17
200 ng	16.36±0.12
20 ng	20.54±0.19
2 ng	23.19±0.21
200 pg	26.44±0.28
20 pg	28.34±0.27
2 pg	30.72±0.14
200 fg	31.84±0.22
Negative control	not determined[a]

[a]no fluorescence was detected after 40 cycles of PCR amplification
SE – standard error

Table 3. Detection of *Xanthomonas hortorum* pv. *pelargonii* in inoculated geranium

Days after inoculation	Percentage of disease incidence	Percentage of detection of the pathogen in inoculated plants	
		real-time PCR	conventional PCR
1	0	0	0
2	0	0	0
3	0	0	0
4	0	28	0
5	42	59	40
6	67	79	64
7	54	91	81
8	100	100	100
9	100	100	100
10	100	100	100

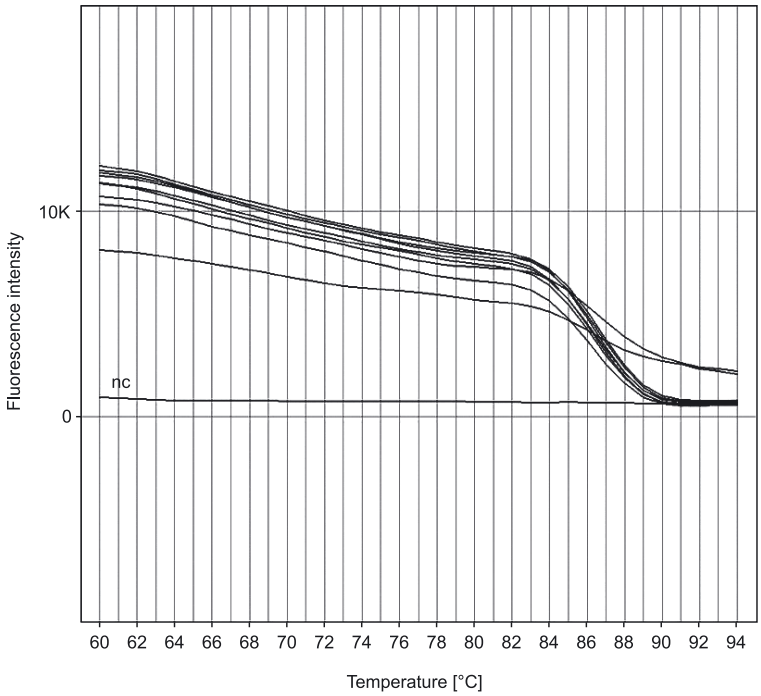

Fig. 2. Real-time PCR melting curve: nc – negative control; remain lines are related to extracted DNA of *Xanthomonas hortorum* pv. *pelargonii* ranging from 2 µg to 200 fg and also positive control

Discussion

There are no reports on the use of real-time PCR for detection of *X. hortorum* pv. *pelargonii* in geranium. This study revealed that real-time PCR using primer pair XhqF and XhqR is reliable for rapid and accurate detection of *X. hortorum* pv. *pelargonii* in asymptomatic infected plants. Thus, real-time PCR is an appropriate method to detect latent infections and can be used for management strategy of the disease. The negative reactions in *in vivo* and *in vitro* conditions suggest that the designed primers are highly specific for *X. hortorum* pv. *pelargonii.* Conventional methods of detection and identification are time consuming, labour intensive, and they lack specificity and sensitivity. Real-time PCR has been developed for high sensitive detection of *Xylella fastidiosa*, *R. solanacearum* and *Agrobacterium* spp. in host tissues (Weller *et al*. 2000; Schaad *et al*. 2002; Weller *et al*. 2002). High efficiency of real-time PCR compared to conventional PCR for detection of plant pathogens was previously reported. Lees *et al*. (2002) reported that real-time PCR was more effective than conventional PCR for the detection of *Rhizoctonia solani* in soil. Safaie Farahani *et al*. (2015) presented that real-time PCR is a more sensitive method than conventional PCR for detection of the causal agent of ratoon stunt in sugarcane. It has been shown that real-time PCR is a better technique compared to conventional PCR for detection of *Colletotrichum coccodes* in soil and potato tubers (Cullen *et al*. 2002). Faghihi *et al*. (2014) applied conventional PCR, nested PCR and real-time PCR for detection of the causal agent of huanglongbing in citrus and showed that real-time PCR is the most sensitive technique. Real-time PCR measures the amount of the product during the exponential phase, whereas conventional PCR computes product during the plateau phase. It is more effective to measure during the exponential phase because measurements taken during the plateau phase are not always correlated with the quantity of starting material. Additionally, real-time PCR avoids post-PCR manipulation such as gels. Though running gel electrophoresis is relatively inexpensive, it is time-consuming and non-automated. However, real time-PCR is expensive and analysis of data is somewhat difficult. Sensitivity is a vitally important factor of a detection method for practical disease diagnosis. Due to its high costs and technical requirements, real-time PCR has not been widely used on a routine basis for detection of plant pathogens. We indicated that real-time PCR assay can be applied to detect latent infection of bacterial blight of geranium caused by *X. hortorum* pv. *pelargonii.* Therefore, real-time PCR assay has good potential for further development as a diagnostic tool for the detection of *X. hortorum* pv. *pelargonii* in commercial applications. In conclusion, conventional and real-time PCR have own advantages and disadvantages. Therefore, the selection of these techniques for diagnosis depends on the factors like disease severity, the goal and available facilities. Our results indicated that conventional and real-time PCR have similar efficiency in symptomatic infected geraniums. Hence, conventional PCR can be used for detection of the bacterium in symptomatic infected geraniums. On the other hand, real-time PCR is more sensitive for detection of the pathogen in asymptomatic infected geraniums.

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References

Anderson M.J., Nameth S.T. 1990. Development of a polyclonal antibody-based serodiagnostic assay for the detection of *Xanthomonas campestris* pv. *pelargonii* in geranium plants. Phytopathology 80: 357–360.

Cullen D.W., Lees A.K., Toth I.K., Duncan J.M. 2002. Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. Plant Pathology 51 (3): 281–292.

Daughtrey M., Macksel M. 1993. Nursery-cultivated *Geranium* spp. as a possible inoculum source for *Xanthomonas campestris* pv. *pelargonii* causing bacterial blight disease of a greenhouse *Pelargonium* crop. Phytopathology 83: 242.

Daughtrey M., Wick R.L. 1993. Vascular wilt diseases. p. 237–242. In: "Geraniums IV. The Grower's Manual" (J.W. White, ed.). Ball Publishing, Geneva, Switzerland, 412 pp.

Faghihi M., Taghavi S.M., Hamzezarghani H. 2014. Comparison of the efficiency of conventional PCR, nested-PCR, real-time PCR and loop-mediated isothermal amplification (LAMP) methods for detection of the causal agent of citrus huanglongbing in Iran. Iranian Journal of Plant Pathology 50: 237–255.

Hodge N.C., Chase A.R., Stall R.E. 1992. Diversity of four species of *Xanthomonas* as determined by cellular fatty acid analysis. Phytopathology 82: 1153.

Lees A.K., Cullen D.W., Sullivan L., Nicolson M.J. 2002. Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. Plant Pathology 51 (3): 293–302.

Manulis S., Valinsky L., Lichter A., Gabriel D.W. 1994. Sensitive and specific detection of *Xanthomonas campestris* pv. *pelargonii* with DNA primers and probes identified by random amplified polymorphic DNA analysis. Applied and Environmental Microbiology 60 (11): 4094–4099.

Nameth S.T., Daughtrey M.L., Moorman G.W., Sulzinski M.A. 1999. Bacterial blight of geranium: a history of diagnostic challenges. Plant Disease 83 (3): 204–212.

NCBI (National Center for Biotechnology Information). Available on: http://www.ncbi.nlm.nih.gov/. [Accessed: December 21, 2015]

Oglevee-O'Donovan W.A. 1986. Production of culture virus-indexed geraniums. p. 119–123. In: "Tissue Culture as a Plant Production System for Horticultural Crops" (R.H. Zimmerman, ed.). Springer, Netherlands, 371 pp.

Safaie Farahani A., Taghavi S.M., Taher-khani K. 2014. Comparison of conventional, nested and real-time PCR for detection of the causal agent of ratoon stunt in Iran. Journal of Plant Pathology 97 (2): 259–263.

Sasser M.J. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Technical Note # 101. Available on: http://natasha.eng.usf.edu/gilbert/courses/nanoscale_biomed/gc_ms/bacteria_gc_1.pdf. [Accessed: December 10, 2015]

Schaad N.W., Opgenorth D., Gaush P. 2002. Real-time polymerase chain reaction for one-hour on-site diagnosis of Pierces disease of grape in early season asymptomatic vines. Phytopathology 92 (7): 721–728.

Sulzinski M.A., Moorman G.W., Schlanhaufer B., Romaine C.P. 1996. Characteristics of a PCR-based assay for *in planta* detection of *Xanthomonas campestris* pv. *pelargonii*. Journal of Phytopathology 144 (7–8): 393–398.

Sulzinski M.A., Schlagnhaufer B., Moorman G.W., Romaine C.P. 1998. PCR-Based detection of artificial latent infections of geranium by *Xanthomonas campestris* pv. *pelargonii*. Journal of Phytopathology 146 (2–3): 111–114.

Tuinier J.E., Stephens C.T. 1989. Use of serology to detect *Xanthomonas campestris* pv. *pelargonii* in aqueous extracts of geranium plants. Plant Disease 73 (11): 875–878.

Weller S.A., Elphinstone J.G., Smith N.C., Boonham N., Stead D.E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied and Environmental Microbiology 66 (7): 2853–2858.

Weller S.A., Simpkins S.A., Stead D.E., Kurdziel A., Hird H., Weekes R.J. 2002. Identification of *Agrobacterium* spp. present within *Brassica napus* seed by TaqMan PCR – implications for GM screening procedures. Archives of Microbiology 178 (5): 338–343.