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Real Time PCR Quantification of *Sclerotium rolfsii* in Chilli Tissue and Soil

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Abstract

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Root rot disease caused by *Sclerotium rolfsii* is one of the important factors limiting chilli production in southern China. Rapid methods of detection and identification of *S. rolfsii* are required for disease prediction and management. Herein, we adapted a four-step real time quantitative PCR method to detect *S. rolfsii* using new designed primer pair S301S/S301A. By using this assay, *S. rolfsii* was detected in ten soil samples and eight plant samples at concentrations as low as 10^{-7} g mycelium/g soil or plant, both with and without typical symptoms, while tissue-culturing method could only detect the fungus in two samples. This rapid and quantitative method is efficient for detection and determination of *S. rolfsii* population in soil and plants.

Keywords: chilli root rot; specific primer pair; pathogen identification; population detection; population determination

Chilli root rot accounts for serious economic losses in India (Kalmesh & Gurjar 2001) and China (Xu 2009). The causative agent, *Sclerotium rolfsii* Sacc., is one of the most destructive soil-borne plant pathogens. It infects more than 500 species of plants in about 100 families in warm moist areas throughout the world (Shew *et al*. 1984). The pathogen can damage either stem or root, produces dark brown lesion at collar region, and causes a plant to wilt, dry up and die rapidly (Punja 1985). In the early stages of root rot in field, white mycelia growing around chilli stem or on the leave debris are not easily distinguishable from other diseases caused by *Phytophthora capsici*, *Rhizoctonia solani*, or *Sclerotinia sclerotiorum*. Thus early diagnosis based on symptoms and conventional tissue-culturing method is difficult for local technicians. Therefore, accurate identification of the pathogen and prediction of the disease are urgently needed. Moreover, the timely and accurate detection and determination of the pathogen population are critical for the study of epidemiology and management of chilli root rot.

PCR methods have been developed to detect *S. rolfsii*. Jeeva *et al*. (2010) designed a primer pair SCR-F/SCR-R to detect *S. rolfsii* in *Amorphophallus paeoniifolius* and infected soil. Cating *et al*. (2012) used a high-fidelity PCR method to detect whether the orchid plants are infected by *S. rolfsii* or not. However, no quantitative PCR (qPCR) program has been developed yet for *S. rolfsii* detection. In this study, we adapted a four-step real time qPCR method (Zhang *et al*. 2004a) to detect and quantify *S. rolfsii* both in chilli tissue and rhizosphere soil, beneficial for a rapid, accurate identification and prediction of the disease.

MATERIAL AND METHODS

Fungi. *S. rolfsii* HN-1-1 was kindly provided by Prof. Zhihuai Liang, Hunan Academy of Agricultural Sciences, Changsha, Hunan, China. Four fungal strains of *Fusarium oxysporum*, *S. sclerotiorum*, *P. capsici*,

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and *R. solani* causing similar disease symptoms in chilli were from our laboratory stock.

Genomic DNA preparation. All tested fungi were cultured on potato dextrose agar (PDA) or oat plates (30 g/l oat and 20 g/l agar) (Qi *et al*. 2001) for 5–7 days at 26°C. Mycelia were harvested, frozen in liquid nitrogen, and grounded into a fine powder. About 50 mg of mycelial powder was subjected to DNA extraction and purification using the E.Z.N.A. Fungal DNA Mini Kit according to the manufacturer's instructions (Omega Bio-Tek, Norcross, USA). The purified genomic DNAs were stored at –20°C before use.

Optimisation of the real time qPCR protocol for S. rolfsii detection. One primer pair S301S (5'-GAACCATCTGTAGTCAGGAGAAATC-3') and S301A (5'-GCCGTAAGGTTGAGAATTTAATGAC-3') was designed using Primer premier 5.0 software (Zhang *et al*. 2004b) with reference to the ITS sequences of *S. rolfsii* (EU863218, GQ148561, and FJ968783) (Jeeva *et al*. 2010). Each 25-µl PCR mixture contained 12.5 µl of 2× Taq PCR StarMix with loading dye (GenStar, Taiwan, China), 1 µl of template DNA, 1 µl of each primer, and 9.5 µl of sterile distilled water. PCR was carried out with a DNA Engine® Peltier Thermo Cycler (Bio-Rad, Hercules, USA) as follows: 2 min at 94°C, 35 cycles of 30 s at 94°C, 50 s at 58°C, 1 min at 72°C, and one final extension of 8 min at 72°C. PCR products were separated on a 1.2% agarose gel and purified using a gel extraction kit (Biomed, Shanghai, China). The purified PCR products were ligated into pMD18-T vector (TaKaRa, Beijing, China), which were further transformed into *Escherichia coli* DH5α competent cells. By screening on LB plates containing 100 µg/ml ampicillin, the positive clones were selected for sequencing.

For the real time qPCR of *S. rolfsii*, a four-step method (Zhang *et al*. 2004a) was used, which included an extra step of 6 s at 77.5°C after the DNA extension step. QPCR was performed using the BioRad iQ5 Multicolor Real Time PCR Detection System (Bio-Rad). Each qPCR mixture contained 1 µl of DNA template, 1 µl (100µM) of each primer, 12.5 µl of SYBR Premix DimerEraser (TaKaRa) containing SYBR Green I, and 9.5 µl of sterile water. In control reactions, 1 µl of sterile water replaced the DNA template.

Real time qPCR assay of S. rolfsii in chilli plants and rhizosphere soil. The positive recombinant plasmids harbouring *S. rolfsii* ITS gene fragments were quantified spectrophotometrically, and then diluted tenfold serially (1.32×10^3–1.32×10^{10} copy/µl) to construct the qPCR standard curve. Sterile distilled water was taken as control.

For soil detection, mycelial suspensions of *S. rolfsii* (10^{-2} to 10^{-7} g/ml) were mixed with 10 g of autoclaved dry soil. *S. rolfsii*-free soil was used as a control. Each 0.5 g of soil sample was subjected to DNA extraction using the Ultra Clean Soil DNA Isolation Kit (Mo-Bio, Carlsbad, USA). The final

Table 1. Detection of *S. rolfsii* in chilli plants and rhizosphere soil by using the real time qPCR assay

Samples	Location	Incidence (%)	Symptoms	Concentration of *S. rolfsii* (g/g)	
				chilli plants ($\times 10^{-7}$)	rhizosphere soil ($\times 10^{-5}$)
Hunan-1	greenhouse	80.0	white mycelia on stem surface	26	78.0
Hunan-2			brown sclerotia on stem and brown stem vascular tissue	67	29.0
Hebei-1	greenhouse	27.6	brown stem and root vascular tissues	trace	0.48
Hebei-2		25.9	stem rot, brown root, and partial stem vascular tissues	1.7	0.41
Hainan-1	greenhouse	82.0	black stem and root	3.0	0.09
Hainan-2			root rot and brown and black stem base	< 1.0	0.18
Hainan-3	greenhouse	23.0	white mycelia on stem base and black stem	< 1.0	440.0
Hainan-4			white mycelia on stem base and black stem	trace	4400.0
Hainan-5			black stem and no white mycelia on stem base	nd	18.0
Hainan-6	field	0.5	dry stem and lightly black lateral root	nd	0.26

nd – no detection

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volume of each DNA sample was adjusted to 50 µl with sterile distilled water, and was subjected to qPCR analysis. A standard curve was generated by plotting the logarithm values of DNA copy number against *S. rolfsii* mycelial weight.

For plant detection, 100 mg of fresh healthy chilli tissue were cut into pieces and mixed with 10 µl of *S. rolfsii* mycelial suspensions (10^{-2} to 10^{-7} g/ml). DNA was extracted using the Plant Genomic DNA Kit (TianGen, Beijing, China) and 100 µl of each sample was used in qPCR. *S. rolfsii*-free healthy tissue was used as a control.

Detection and quantification of S. rolfsii in chilli field samples. Nine greenhouse and one field chilli plants in addition to their rhizosphere soils were collected from Hebei, Hunan, and Hainan provinces as listed in Table 1. All plant tissues close to the decaying stem areas were collected and separated into two sections. One section was used for pathogen isolation with the tissue-culturing method (Burgess *et al.* 2008) on PDA plates containing 100 µg/ml of each ampicillin and streptomycin, and the isolation frequency was recorded. The other plant section and rhizosphere soil were subject to qPCR analysis as described above.

RESULTS

Optimised protocol for real time qPCR assay of S. rolfsii. In this study, S301S/S301A was identified as a highly specific and efficient primer pair. No DNA fragment was amplified from other tested species using this primer set (Figure 1). The specific DNA fragment was approximately 300 bp (accession No. KC676789), and shared 99% identity to the *S. rolfsii* ITS sequences GQ148561 and FJ968783. Using this primer set, we compared the efficiency of traditional three-step real time PCR (Figure 2A, B) for *S. rolfsii* detection with that of optimized four-step one (Figure 2C, D) by including an extra step after the DNA extension step. Melt curves of primer dimer (A-1 and A-2) and PCR amplicons (B-1 and B-2) were both detected (Figure 2A, C), but their corresponding amplification curves were different (Figure 2B, D). The three-step qPCR program gave two amplification curves, AA-1 for primer dimer and BB-1 for amplicons (Figure 2B). The fluorescence signals were disturbed by the amplification of the primer dimer (curve AA-1). In contrast, a single perfect amplification curve (BB-2 in Figure 2D) was detected under the conditions of 81 cycles from 55°C to 95°C with a temperature increase of 0.5°C per 10 seconds. Finally, a four-step real time PCR protocol for *S. rolfsii* detection was optimised as follows: 30 s at 95°C, 41 cycles of 95°C for 10 s, 57°C for 30 s, 72°C for 1 min, and an extra holding step of 6 s at 77.5°C.

Real time qPCR standard curves for S. rolfsii detection in chilli plants and soil. The specific DNA fragment of *S. rolfsii* was successfully amplified from the positive recombinant plasmids. Real time qPCR was conducted with the plasmid DNA as template. Based on the C_t values and the copy numbers of plasmids, a linear standard curve, $Y = -3.238X + 44.812$, was constructed, where X is the DNA copy number and Y is the C_t value, respectively.

With reference to the plasmid DNA amplification curve, the C_t values of *S. rolfsii* mycelia in soil and plant samples were converted into DNA copy numbers by using the linear regression equations $Y = 0.997X + 1.571$ and $Y = 0.987X + 3.942$, where X is the logarithm of *S. rolfsii* mycelial weight in 10^{-2} g soil sample or 10^{-3} g tissue sample, and Y is the logarithm of copy number (Figure 3). The high regression coefficients (0.9929 for soil samples and 0.9992 for plant tissues, respectively) allowed for the reliable and accurate detection of *S. rolfsii* in soil and tissue samples at concentrations as low as

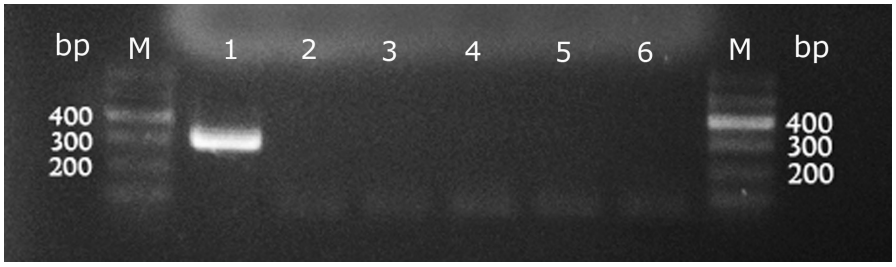

Figure 1. Agarose gel electrophoresis of the PCR products amplified with the primer pair S301S/S301A

Lane M = DNA standard markers (100–600 bp, TianGen, Beijing, China); lane 1 = specific DNA fragment (about 300 bp) amplified from the genomic DNA of *S. rolfsii*; lanes 2–5 = no objective DNA fragment amplified from the DNA templates of *S. sclerotiorum*, *P. capsici*, *F. oxysporum,* and *R. solani*, respectively; lane 6 = negative control of distilled water

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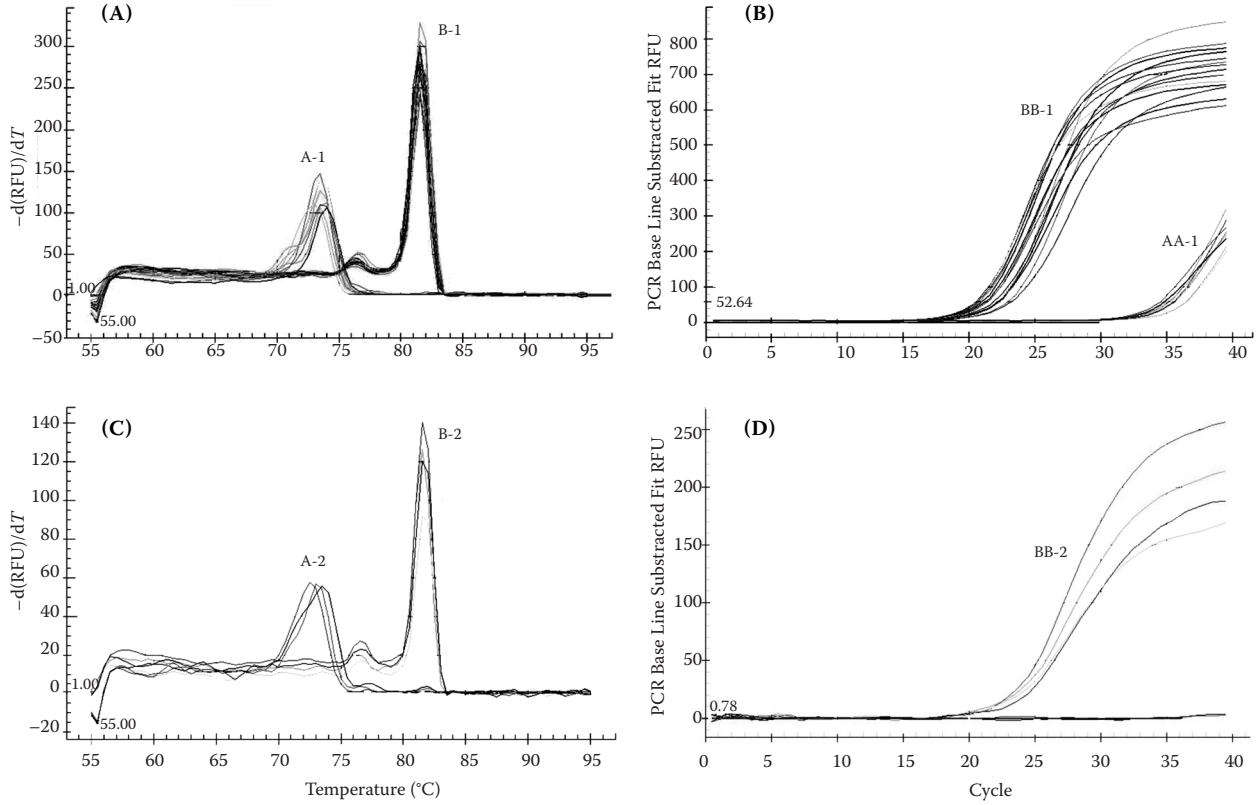

Figure 2. Efficiency comparison of the three- and four-step real time qPCR programs. A, B = melt curve and amplification curve of three-step real time qPCR program; C, D = melt curve and amplification curve of the four-step real time qPCR program including an extra step of 6 s at 77.5°C after the extension step

Curves A-1 and A-2 are the melt curves of primer dimer, and curves B-1 and B-2 are the melt curves of amplicons. Curve AA-1 is the amplification curve of primer dimer, and curves BB-1 and BB-2 are the amplification curves of amplicons

10^{-7} g mycelia/g. It means that target DNA could be detected at a limit of 1064 copies/g soil or plant, which was equivalent to 6.6 pg/ml pure DNA.

Detection of S. rolfsii in chilli plants and in field soil. In comparison to qPCR analysis, *S. rolfsii* was also isolated and identified using the tissue-culturing method (Table 1). Fungal strains were only isolated from three samples (Hunan-1, Hunan-2, and Hainan-2), and one fourth and one third of Hunan-1 and Hunan-2 isolates were identified to be *S. rolfsii*, respectively, while other isolates in these two samples were identified to be *Fusarium*. In the plant samples of Hainan-2, white mycelia grew from the chilli tissues, but it was difficult to identify to the genus level based on morphological characteristics. Using real time PCR, we detected *S. rolfsii* in all soil samples at concentrations of 10^{-7} to 10^{-2} g/g soil, and in most chilli samples at 10^{-7} to 10^{-6} g/g chilli tissue (Table 1). The amounts of *S. rolfsii* in rhizosphere soil were high in four samples (Hunan-1, Hunan-2, Hainan-3, and Hainan-4), where chilli stem bases were covered with white mycelia or sclerotia and the incidence of root rot reached 80.0 and 23.0%, respectively. The *S. rolfsii* amounts in chilli tissues varied a lot, being high in samples of Hunan-1 and Hunan-2, moderate in Hebei-2 and Hainan-1, and low in Hebei-1, Hainan-2, Hainan-3, and Hainan-4. No *S. rolfsii* was detected in the samples of Hainan-5 and Hainan-6.

DISCUSSION

Chilli plants infected by *S. rolfsii* can wilt and die in several days (Jenkins & Averre 1986). Therefore, rapid and accurate detection of *S. rolfsii* in soil or early diagnosis of the disease becomes urgent for efficient disease control. In order to set up a rapid qPCR method for detection of *S. rolfsii*, a specific primer set and an optimised protocol have been developed in this study. Considering the requirement of qPCR with SYBR Green I as the fluorescence dye (Primer and probe design – PCR and quantitative PCR 2010; http://

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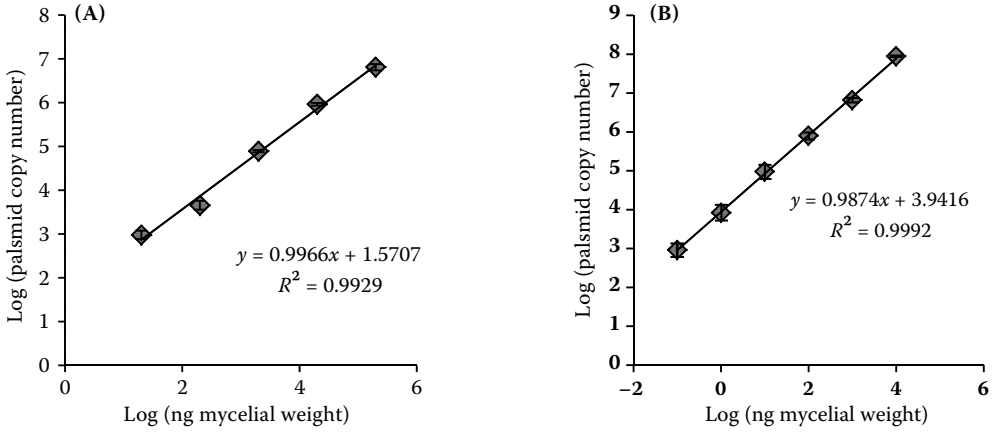

Figure 3. Real time PCR standard curves for qualification of the mycelial amounts of *S. rolfsii* in chilli rhizosphere soil (A) and stem tissues (B)

The copy numbers were transformed from the C_t values based on the plasmid standard curve, and plotted against the logarithm of mycelial weight in 1 µl of pure DNA sample

wenku.baidu.com/view/2a1807fe04a1b0717fd5ddf0.html), we had to design a primer set that generates amplicons shorter than 300 bp. Thus the primer set specific for *S. rolsfii* designed by Jeeva *et al.* (2010) is unsuitable, which amplifies a fragment of 540 bp. Based on the ITS sequences of *S. rolsfii*, we designed a primer set S301S/S301A. It has excellent specificity as shown in Figure 1, but forms disturbing dimers. In order to eliminate its effects, we adapted a four-step real time PCR program (Zhang *et al.* 2004a) by including an extra step at a temperature between the T_ms of primer dimer and PCR amplicon after the DNA extension step for fluorescence signal readings. At this temperature, the target DNA fragments were still double-stranded, while the primer dimers melted and produced no fluorescence signal. As a result, the fluorescence signals were specific, and this assay was effective to detect *S. rolfsii* both in soil and in chilli tissue samples.

For accurate disease prediction, a correlation between the pathogen density and disease incidence should be constructed. The correlation between the density of sclerotia and disease development was developed by Punja (1986). However, the germination of sclerotia needs appropriate temperature and humidity (Jenkins & Averre 1986). Thus, the amount of mycelia instead of sclerotia was selected for quantification of the pathogen in this study. By using the qPCR method adapted in this study, *S. rolfsii* was successfully detected from chilli plants or field soil. Its density was correlated with the disease incidence and severity. The results revealed the application potential of this qPCR method for prediction of chilli root rot.

The detection limit of the qPCR assay developed in this study was 1064 copies/g plant or soil, i.e. 6.6 pg/ml pure DNA. This detection method was more rapid and sensitive than the culturing method, but it should be further improved, as high-fidelity PCR methods can detect 207 (Cating *et al.* 2012) plasmids in the presence of plant DNA or 25 pg pathogen/g soil (Jiménez-Fernández *et al.* 2010). In further studies, we will combine the qPCR assay developed in this study with fluorogenic probes (Yin *et al.* 2001) or nested PCR (Landa *et al.* 2013) to improve the detection sensitivity.

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