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A real-time quantitative PCR assay for specific and sensitive detection of *Phytophthora infestans* on tomato

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Late blight, caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary, is the most disastrous disease of tomato and potato worldwide (Cooke *et al*. 2004). It has emerged as a serious threat to tomato cultivation in Karnataka since 2008. The disease is likely to spread to other states from Karnataka through seeds and seedlings. Early accurate detection and identification of *P.infestans* in tomato is essential for containing the movement of spread and for formulating effective disease management strategies. Conventionally, detection and identification of *P.infestans* has been relied on morphological criteria which is time-consuming and requires considerable mycological expertise (Erwin and Ribeiro 1996). Successful isolation of *P.infestans* from infected tomato plants require specific protocols with rye agar medium and direct isolation from symptomatic tissue is a difficult task (Tumwine *et al*. 2000). The plasticity in morphology and difficulty in isolation and culturing necessitated for a sensitive and reliable method of detection in order to prevent its spread and improve prophylaxis, which could be an effective management practice. A conventional PCR based assay has been successfully employed for detection of the *P. infestans* on tomato and potato (Trout *et al*. 1997) but it is gel based, time consuming and not useful for routine quantification. In addition to detection and identification, rapid assay and pathogen quantification is very much required for determining the necessity, and the extent, of appropriate control strategies. Real time PCR assays, using SYBR Green I, have been a powerful development with regard to early detection and quantification of fungal plant pathogens (Lievens *et al*. 2006, Alaei *et al*. 2009) in plant and soil samples. Real-time PCR differs from conventional end-point PCR by the measurement of the amplified PCR product at each PCR cycle. Since the development of the exponential phase of the reaction is monitored, real-time PCR allows accurate template quantification (Mackay *et al*. 2002). Real time PCR is highly sensitive and can detect the pathogen even if DNA is present in minute quantities. The objective of this study was to develop a sensitive and quantitative method for rapid detection of *P. infestans* in infected tomato material and compare sensitivity of this method with already available conventional PCR based assay.

P. infestans Isolate (PIT2) was obtained from naturally infected tomato foliage, during 2011 at Indian Institute of Horticultural Research, Bangalore as per the protocol described by Tumwine *et al*. (2000) and maintained on rye agar A at 18°C in darkness for 12 days. Zoospores were released by incubating mycelial pieces (2 cm × 2 cm) in sterile distilled water at 4°C for 1 hr. Zoospores were collected by passage of the suspension through Whatman No. 541 filter paper to remove chlamydospores and mycelial fragments and counted using a haemocytometer. Suspensions were adjusted to a concentration of 3×10^5 zoospores.

Fully matured leaves from 35 days old tomato plants var Arka Vikas were excised at the petiole base, washed thoroughly with sterile distilled water and sprayed with the zoospore suspension of *P.infestans* isolate (PIT2) (3×10^5 zoospore/ml) and placed in moist chambers containing 98% humidity and incubated at 18ºC. Leaves sprayed with water served as healthy controls. After 6 days of inoculation, necrotic leaf tissue was excised. To know the detection limit of target DNA in plant tissue, serially diluted the 100mg of infected leaf material with 900 mg healthy plant material and created plant material containing 100% to 0.001% of infected material. Three replicate dilution series were prepared. DNA was extracted from 100 mg of each sample using ZR Plant/ seed DNA kit (Zymo Research Corporation, USA) as per recommendations. To determine the target DNA concentration at various stages of the infection process, the conventional and real-time PCR method was used with DNA extracted from plant material inoculated with *P. infestans* and harvested at 0, 1, 2, 3, 4 and 5 days after inoculation.

DNA was extracted from 12 days old fungal mycelium grown in pea broth at 18°C according to the procedure

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described earlier (Chowdappa *et al*. 2003). The ITS region of ribosomal DNA from *P.infestans* isolate (PIT2) was amplified with PINF (Trout *et al*. 1997) and ITS5 (White *et al.* 1990) primer. The resulting PCR product was cloned into plasmid and sequenced to confirm that it has 100% homology to rDNA sequence of *P.infestans* available at NCBI. The genomic DNA (gDNA) and plasmid DNA (pDNA) was quantified using Nano drop spectrophotometer and serially diluted to obtain concentrations from 500ng to 5pg/µl.

Conventional PCR was performed according to the procedure of Trout *et al*. (1997) using PINF and ITS5 primers. Real-time PCR amplifications were done in glass capillaries in a total volume of 20 µl using the intercalating dye SYBR Green I on a Light cycler 2 (Roche Diagnostics Corp., Indianapolis, IN, USA). Each reaction contained 1 µl of the target DNA extract, 10 µl of the SYBR Green I PCR master mix 2x (Roche Diagnostics Corp., Indianapolis, IN, USA), 1 µl of each primer (50 pM) PINF and ITS5 and 7 µl sterile distilled water. Thermal cycling conditions consisted of initial denaturation for 10 min at 95°C followed by 40 amplification cycles of 10s at 95°C, 30 s at 55°C, 30s at 72°C. Fluorescence (530 nm) was detected at the end of the elongation phase for each cycle. To evaluate amplification specificity, melting curve analysis was performed at the end of each PCR run. A melting curve profile was obtained by heating the mixture to 95°C for 10s, cooling to 70°C for 20s and slowly heating to 95°C for 20s at 0.05°C/s ramp rate with continuous measurement of fluorescence at 530 nm. Crossing point (Cp) values, which are inversely proportional to detected DNA content, were calculated using Light Cycler software 2.0. Reactions were run in triplicate to minimise the error due to handling.

Standard curves were generated by plotting the logarithm of DNA concentrations against the Cp values collected by the Light Cycler software 2.0 and regression equation was calculated. Unknown samples were quantified from measured Cp values by interpolation using the regression equation.To assess the sensitivity of the detection of *P. infestans*, DNA dilution series resulting in 500ng to 1pg/µl of DNA from isolate *P. infestans* PIT2 per PCR reaction was subjected to conventional and real-time PCR analyses. To assess the accuracy and repeatability of the real-time PCR setup, three independent real-time PCR reactions were performed. To find out the possible interference of tomato DNA or other non-target DNA from other fungal species, *Alternaria solani, A. alternata, Athelia rolfsii, Botrytis cinerea, Fusarium oxysporum* f. sp. *lycopersici* and *Phytophthora parasiticae* associated with tomato on accurate detection and quantification of target pathogen DNA, 50 and 5 pg/µl target DNA were added to 20 pg/µl genomic DNA from a healthy tomato or other fungal species. For all samples, three replicates were analyzed. Finally, the assays were validated using naturally infected samples collected from 15 different localities in Bangalore rural, Chikkaballapura and Kolar districts of Karnataka.

The ITS sequence from PIT2 isolate has been deposited in the NCBI, as accession number HQ191483. Further, identity of the fungal isolate was confirmed by obtaining product of 600bp in size with species-specific PCR using PINF/ITS5 primer pair with DNA isolated from pure mycelium (Fig 1a). Conventional and real time PCR of *P. infestans* were compared using a 10 -fold dilution series of gDNA or pDNA. The detection limit in conventional PCR is 50pg of DNA (Fig 1a) and 30 zoospores. There is no amplification if the template DNA used is 5 or below 5pg. The limit of detection was 5pg of DNA (Fig 1b) and 3 zoospores in case of real time PCR. The standard curves obtained from separate real-time PCR assays and different dilution series of g DNA and p DNA were highly similar: the coefficient of variation between the Cp values was smaller than 5% at all template levels. Highly linear relationships (R^2 = 0.9843 for g DNA and R^2 = 0.9933 for pDNA) were observed between the Cp value and the log of the DNA concentration in each replicate. The slope of the standard curves was significantly more or less similar (P> 0.05) for p DNA template (–4.32 ± 0.18 average ± standard deviation) versus gDNA template (–3.53 ± 0.20) (Fig 2a), which indicates that pDNA can be used reliably for the preparation of standard curves and thus the quantification of *P. infestans* in unknown DNA samples. When real-time PCR was conducted on pDNA

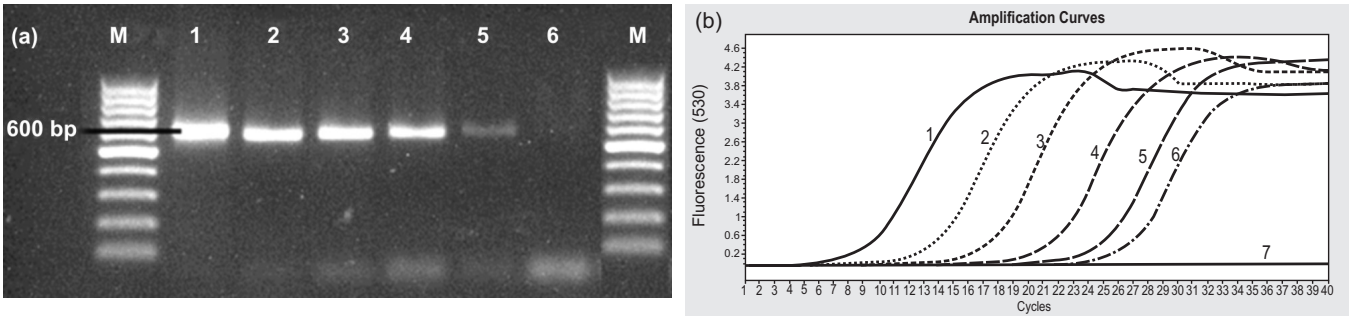

Fig 1 (a) The amplified products of serially diluted gDNA with Conventional PCR. (b) The amplification curves of 10-fold serially diluted gDNA with Real time PCR. Lane M - 100bp ladder; Lane1-500ng; lane2-50ng; Lane3-5ng; Lane4-500pg; Lane5-50pg; Lane6-5pg.

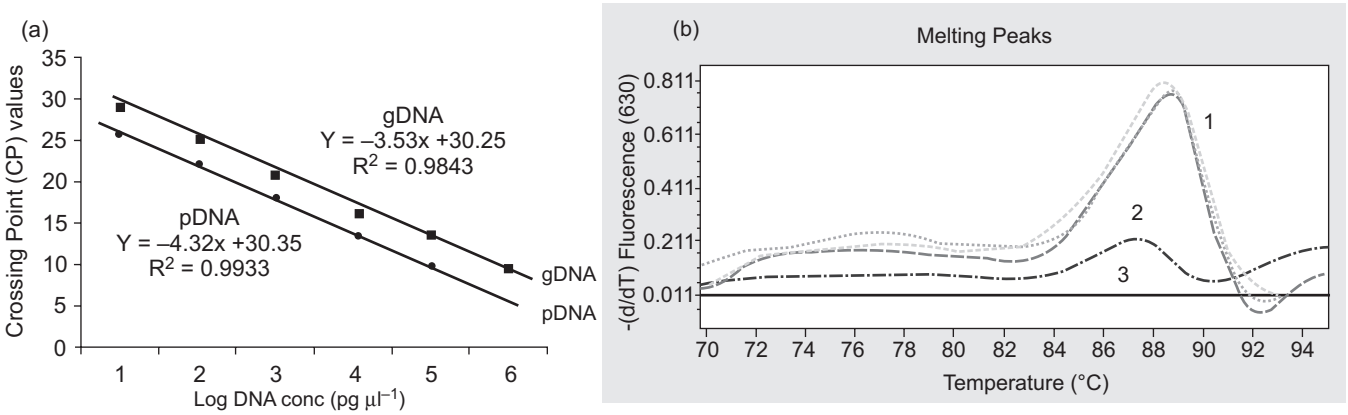

Fig 2 (a) Standard curves used for the quantification of target DNA in tomato samples. Standard curves were obtained with 10-fold series of plasmid and genomic DNA of *P.infestans.* Data represent means of three values (error bars, representing standard errors, are too small to be displayed graphically). (b) Melting curve analysis with the corresponding melting temperatures of the PCR assays. 1. Five melting peaks representing target g DNA, target p DNA, target g DNA spiked with tomato DNA, target g DNA and DNA of *P.infestans*, 2. non-templated control, 3.Water control.

and gDNA of *P. infestans* that was diluted with genomic DNA of tomato or other tomato pathogens such as *Alternaria solani, A. alternata, Athelia rolfsii, Botrytis cinerea, Fusarium oxysporum* f. sp. *lycopersici* and *Phytophthora parasitica*, no significant difference was observed between the standard curves (P> 0.05) or the correlation coefficients (R^2 =0.99), suggesting no interactive effect of the host DNA or other tomato pathogens with the PCR reactions.

Crossing point (Cp) values in samples from target gDNA and pDNA, infected tomato and non-target fungal material were more or less similar and ranged from 25.48 to 26.34 for 5pg. None of the other fungal cultures associated with tomato yielded Cp values with real-time PCR assays, indicating that the assays were highly specific. Although SYBR green dye could not discriminate between the different dsDNA molecules in a PCR reaction but different PCR amplification products can be accurately distinguished by melting curve temperature analysis (T_m) of the final amplicons (Ririe *et al*. 1997, Schena *et al*. 2004). Melting curve analysis revealed that the melting temperatures of the PCR products and pDNA or gDNA of *P. infestans* or DNA of symptomatic leaves were uniform for all template concentrations, indicating the specificity of the amplification process. The melting peaks were within the range of 88.0°-89.0°C (Fig 2b). No amplification could be detected using template DNA from any of the non-target fungal species tested. Using primers PINF and ITS 5 primer pair and Tm analysis, no peaks that would suggest the production of non-target amplicons, even at low target concentration were observed. Previous studies have used real-time PCR detection of fungal plant pathogens through SYBR Green I (Lievens *et al*. 2006, Frederick *et al*. 2002). By using conventional PCR, the pathogen could be detected only 4 days after inoculation while in real-time PCR pathogen could be detected 2 days after inoculation. Characteristic visual symptoms appeared at 6 days of inoculation. When using infected plant material in a dilution series with healthy plant material, the pathogen could be detected with conventional PCR, when at least 0.1% of the plant material was infected. With real-time PCR, the detection limit was the 0.001% infected plant material. Presence of pathogen in naturally infected leaf, stem and fruit samples of tomato collected from 15 different localities in Bangalore Rural, Chikkaballapura and Kolar districts of Karnataka during 2011 was detected by real time PCR assays. Presence of the *P. infestans* in naturally and artificially inoculated tomato leaf, stem and fruit samples were further established by successful isolation of fungus on selective rye agar A medium. No product was detected in healthy samples. The method presented here can detect early infection stages at low disease incidence and a detection limit of approximately 5 pg, which is highly comparable to results reported for other fungi such as *Puccinia horiana in Chrysanthemum x morifolium* (Alaei *et al*. 2009) and *Tilletia indica* and *T. walker* in wheat (Frederick *et al*. 2002).

SUMMARY

A simple, reliable and rapid real-time PCR-based assay using SYBR Green I was developed for detection of *P. infestans* in tomato in the present study. This method has the advantage of the quantitative detection of the *P.infestans* in tomato samples after a single PCR-run. By using this real-time PCR method, it is possible to detect and quantify the pathogen during early stages of infection. This method can be successfully employed not only for diagnosis purpose in plant quarantine clinics but also useful in epidemiological studies and to reduce risk of development of fungicidal resistance.

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