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Detection and quantification of *Alternaria solani* in tomato by real time PCR

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ABSTRACT

A conventional and real-time PCR assays using SYBR Green for the detection and quantification of *A. solani* have been developed and validated. A primer set (ALP and ITS4) designed from the ITS region of *A. linicola/ A. solani complex,* yielded a 536 bp product when DNA from 38 isolates of *A. solani* were amplified. No product was amplified from *A. alternata, A. brassicae, A. brassicicola, A.helianthi, A. porri, A. sesami, A.carthami, A.ricini, Colletotrichum gloeosporioides, C. capsici, C. falcatum, Cercospora canescens, C. capsici, Phytophthora infestans, Sclerotium rolfsii, Fusarium equiseti, F. oxysporum, Rhizoctonia solani, Phoma exigua, Curvularia spp* and *Drechslera*. In addition, ALP/ITS4 primers were successfully utilized in real-time PCR assays of *A. solani*. The efficiency of conventional and real-time PCR assays was compared. The conventional PCR was able to detect the pathogen on symptomatic artificially infected tomato plants 5 days after pathogen inoculation. The detection limit was 100 conidia and 10 pg of DNA in the case of conventional PCR. Real-time PCR exhibited a detection limit 10 times lower (10 conidia, 10fg of DNA). The application of real time PCR assay for rapid detection of *A.solani* in infected tomato plant material is discussed.

Key words: *Alternaria solani*, molecular diagnosis, Real Time PCR, early blight

INTRODUCTION

Early blight caused by the nectrotrophic Hyphomycete *Alternaria solani* (Ellis & Martin) Sorauer, is a disease with huge economic impact in many tomato (*Lycopersicon esculentum* Mill.) growing areas around the world and can reduce yield by 79% (Gwary and Nahunnaro 1998). *A. solani* produces a wide range of symptoms at all stages of tomato plants, causing early blight, collar rot, stem lesions and fruit rots (Chaerani and Voorrips, 2006). The fungus is seed borne (Khulbe and Sati,1987) and also overwinters in plant debris in the form of mycelia, conidia and chlamydospores (Patterson,1991) and other alternate hosts like potato, egg plant and pepper (Ellis and Gibson, 1975) providing a potential primary source of inoculum for early blight epidemics. Detection of the pathogen inoculum sources, particularly in seed and seedling, prior to planting could prevent introduction of infected material into fields, which could be an effective management practice. The currently available technique to detect *A.solani* in tomato seed and infected plant material is based on culturing on agar media and further its morphological characterization (Rotem, 1994), which demands specialized mycological expertise. *A. solani* can be artificially grown in various culture media, but it does not readily sporulate *in vitro*, making identification difficult based on morphology (Rotem, 1994). Further, numerous saprophytic *Alternaria* spp. commonly are found on tomato seed, complicating the identification process. An alternate rapid and accurate method for specific detection of *A.solani* in seed and other plant material is required.

Conventional diagnostic PCR assays have been successfully employed in seed health testing (Guillemette *et al*, 2004; Ioos *et al*, 2009). Tedious and time-consuming post-amplification procedures such as gel electrophoresis and detection by gel documentation and cross contamination limiting large-scale applications of conventional PCR for routine fungal pathogen diagnosis. In addition to detection and identification, pathogen quantification is an important aspect with respect to plant disease management, since it provides the information required for determining the necessity, and the extent of appropriate control strategies.

In recent years, real time PCR based techniques have emerged as robust tools for diagnosis and quantification of

seed borne pathogens and have contributed greatly to plant disease management (Guillemette *et al*, 2004; Lievens *et al*, 2006; Alaei *et al*, 2009; Debode *et al*, 2009). Real time PCR offers several advantages compared to conventional PCR based detection of plant disease diagnosis: increased sensitivity, no post PCR processing and allows quantification of target DNA (Schena *et al*, 2004). However, no report is available on the PCR detection of *A.solani* in tomato except few publications on molecular diversity (Weir *et al*, 1998; Martinez *et al*, 2004; Lourenco *et al*, 2009). To our knowledge, this is the first report concentrating on the molecular detection and quantification of *A.solani* directly from tomato tissue. The objective of this study was to develop sensitive and specific quantitative real-time PCR assay for detection of the *A.solani* in seed and other plant material of tomato and compare its sensitivity to a conventional PCR assay.

MATERIAL AND METHODS

Alternaria solani isolate (OTA 5) (NCBI accession no HQ270458), obtained from naturally infected tomato foliage, during 2011 at ICAR-Indian Institute of Horticultural Research, Bengaluru, was used in the present study. The mycelium was grown in potato dextrose broth at 25 ±1°C, washed with sterile distilled water to remove traces of medium and transferred to filter paper to damp dry. DNA was extracted from the mycelium according to the protocol described earlier (Chowdappa *et al*, 2003). DNA from conidia was extracted using NaOH lysis method described by Nam *et al* (2007). The DNA pellet was re-suspended in 30 to 50 µl of 10mM Tris-EDTA buffer, pH 8.0 and stored at "20°C. The ITS region of r DNA was amplified with ITS1 and ITS4 primers (White *et al*, 1990). The resulting PCR product was cloned into *E.coli* DH5α and sequenced the plasmid inserts to confirm that it has 100% homology to rDNA sequence of *A. solani* available at GenBank. The genomic DNA (gDNA) and plasmid DNA (pDNA) was quantified using Nano drop spectrophotometer and serially diluted to obtain concentrations from 100ng to 10fg/µl.

Conidial suspension (2x 10^6 conidia/ml) of *A.solani*, derived from artificially inoculated tomato fruits var. Arka Vikas, was used as source of inoculum. Fully matured leaves from 40 days old tomato plants var. Arka Vikas were excised at the petiole base and washed thoroughly with sterile distilled water, damp dried and point inoculated with 20µl of conidial suspension of *A. solani* and placed in moist chambers containing 95% humidity at 25ºC under photoperiod of 12h light. Leaves inoculated with water served as control. The necrotic tissue with abundant conidia were excised after 7 days of inoculation and considered as 100% infected plant material. To estimate the detection limit of target DNA in leaf tissue, 100mg of 100% infected plant material was serially diluted with 900mg of healthy material and created plant material containing 100% to 0.001% infection. Three replicate of dilutions series were prepared. The healthy seeds var. Arka Vikas were artificially inoculated with 2×10^6 conidia of *A. solani* following the procedure described by Ioos *et al* (2009). Infection of tomato seeds by *A.solani* was further confirmed by inoculating 100 inoculated seeds randomly on PDA at 25 ±1°C. To know the detection limit of target DNA in seed, seedlots with infestation levels ranging from 10.0% to 0.1% were created by mixing 900 healthy seeds and 100 infected seeds (10% infection), 990 healthy seeds and 10 infected seeds (1% infection) and 999 healthy seeds and 1 infected seeds (0.1% infection). DNA was extracted from 100mg of plant sample or 25 number of seeds using ZR Plant/seed DNA kit (Zymo Research Corporation, USA) as per manufacturer's recommendations. To determine the target DNA concentration at various stages of the infection process in the leaf, the conventional and real-time PCR method was used with DNA extracted from plant material inoculated with *A. solani* and harvested at 0, 1, 2, 3, 4 and 5 days of post inoculation.

Conventional PCR

Conventional PCR was performed according to the method of Chowdappa *et al* (2003) using ALP (5'-GGCACCTCCCGGGGTGGC-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') primers (McKay *et al*, 1999). PCR was performed in 50µl reaction containing 1µl template DNA, 5µl of 10x PCR buffer, 40.7µl of sterile distilled water, 1µl of 10mM dNTP's, 1µl of each 10pmol each *A. solani* specific primer and 0.25µl of Taq DNA polymerase (5U/µl). PCR amplification was performed in an Eppendorf master cycler by 34 cycles of denaturation at 94ºC for 60s, annealing at 55ºC for 60s, and extension at 72ºC for 1.5min with an initial denaturation of 5min at 94ºC before cycling and a final extension step at 72ºC for 5min. To determine if the correct sized PCR product was amplified, 5 ìl of the PCR product were electrophoresed in 2% agarose gel at 90V for 1h in Tris-Borate-EDTA (TBE) buffer and visualized under UV after staining with ethidium bromide (0.5 µg/ml). To check the specificity of the primer pairs, ALP and ITS4, DNA from *Alternaria solani, A. alternata, A.brassicae, A. brassicicola, A.helianthi, A.porri, A.sesame, A.carthami, A.ricini, Colletotrichum*

gloeosporioides, C.capsici, C.falcatum, Cercospora canescens, C.capsici, Phytophthora infestans, Sclerotium rolfisi, Fusarium equiseti., F.oxysporum, Rhizoctonia solani and *Phoma exigua, Curvularia spp and Drechleria* species associated with tomato and other crops has been tested and it showed amplification of *A.solani* and not from other inter and intra related species.

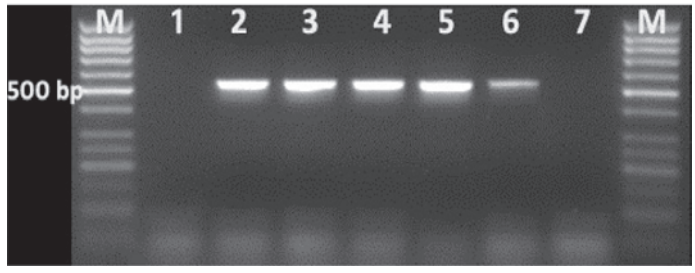
Real time PCR

Real-time PCR was performed with the SYBR® Green (Roche Diagnostics Corp., Indianapolis, IN, USA) in 20 µl reactions contained 1 µl of the target DNA extract, 10 µl of the SYBR Green I PCR master mix 2x, 1 µl of each primer (50 pM) ALP and ITS4 and 7 µl sterile distilled water. Amplification and detection of fluorescence (530nm) was carried out using Light cycler 2 (Roche Diagnostics Corp., Indianapolis, IN, USA). Thermal cycling conditions consisted of initial denaturation for 10min at 95°C followed by 40 amplification cycles of 10s at 95°C, 30 s at 55°C, 30s at 72°C. Fluorescence 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 530nm. 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

The standard curve was generated using tenfold dilution series of target pDNA and gDNA amount (1ng/µl to 10 fg/µl) from *A. solani* isolate against the Cp value exported from the Light cycler 2 Real-Time Detection System. For all the concentrations, three replicate real–time PCR reactions were conducted to establish linear regression curves between threshold cycle (Cp) and the logarithm of the template DNA concentration. The amount of DNA for unknown samples was extrapolated from the Cp value and the value obtained from the standard curve. In order to know the interference of non-target fungal DNA in the real-time assay, the same serial dilutions of *A. solani* isolate OTA5 DNA (i.e., ranging from 1ng to 10 fg/µl) were added to 10 pg/µl of genomic DNA from *A. alternata, A. brassicae, A. brassicicola, A. helianthi, A. porri, A. sesami, A. carthami, A. ricini, Colletotrichum gloeosporioides, C. capsici, , Cercospora capsici, Phytophthora infestans, Sclerotium rolfsii, Fusarium equiseti, F. oxysporum, Rhizoctonia solani, Phoma exigua, Curvularia spp* and *Drechslera* associated with tomato or healthy tomato DNA prior to amplification. For all samples, three replicates were analyzed. Finally, the assays were validated using naturally infected samples collected from 30 different localities in Bengaluru rural, Chikkaballapura, Hassan, Kolar and Tumkur districts of Karnataka, Chittoor district of Andhra Pradesh and Coimbatore district of Tamil Nadu.

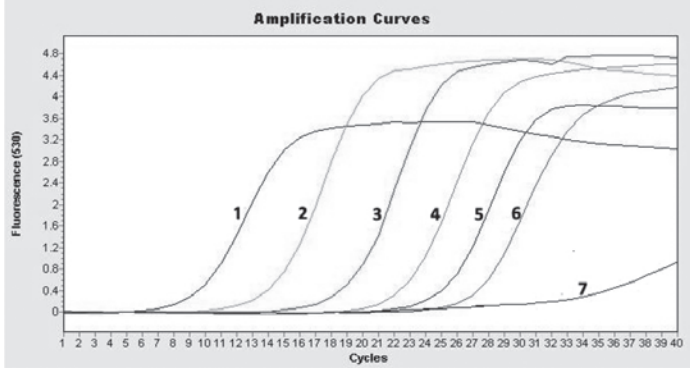
RESULTS AND DISCUSSION

The primer pair, ALP and ITS4 selectively amplified DNA from *A. solani* isolate OTA5, producing amplicon of 537bp and no amplification of DNA from other fungal isolates was detected. Sequencing of amplified fragment showed a nucleotide sequence exhibiting 100% identity with ITS gene of *A. solani*. The detection limit in conventional PCR is 10pg of DNA (Fig.1) and 100 conidia. The limit of detection was 10fg of DNA (Fig. 2) and 10 conidia in case of real time PCR. A characteristic sigmoidal amplification curves were obtained for the different concentrations of target DNA when fluorescence intensity at 530nm was plotted against the cycle number (Fig. 2). The standard curves from



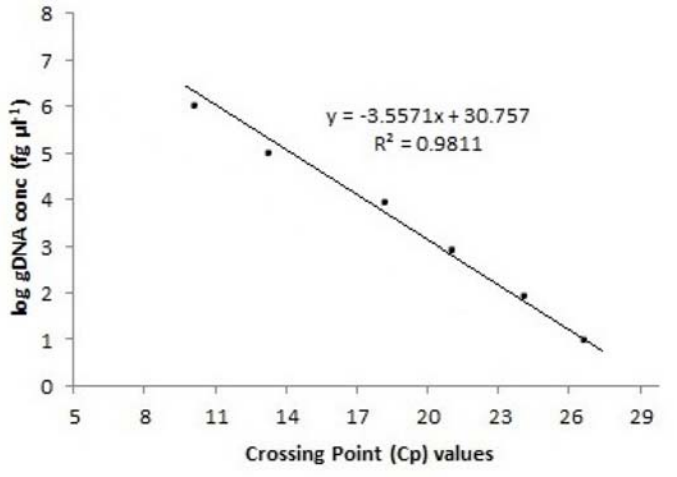
Lane M - 50bp ladder; Lane 1- control; Lane 2-100ng; lane 3-10ng; Lane 4-1ng; Lane 5-100pg; Lane 6-10pg; Lane 7-1pg

Fig. 1. Amplified products of serially diluted gDNA using conventional PCR



Curve1- 1ng; Curve2-100pg; Curve3- 10pg; Curve4- 1pg; Curve5- 100fg; Curve6- 10fg; Curve7- Control

Fig. 2. Amplification curves of 10-fold serial dilutions of target gDNA of *A. solani* using real time PCR



Standard curves were obtained with 10-fold series of genomic DNA of *A. solani*. Data represent means of the three independent reactions.

Fig.3. Standard curves for the quantification of target gDNA in tomato samples

simultaneous real-time PCR reactions were y = "3.557x + 30.757 (R^2 =0.9811) for gDNA and y = "3.554x + 32.377 (R^2 =0.9916) for pDNA which 'y' represents the log concentration of the target DNA and 'x' represents the Cp value. The standard curves obtained from the separate real-time PCR assays and five different dilution series of gDNA and pDNA were highly similar: the coefficient of variation between the Cp values was smaller than 5% at all template levels. Highly linear relationships (R^2 =0.99) were observed between the Cp value and the log of the DNA concentration in each replicate. The slope of the standard curves was not significantly different (P>0.05) for pDNA template ("3.557 ± 0.120) versus gDNA template ("3.558±0.230), which implies that pDNA can be used reliably for the assembly of standard curves and thus the quantification of *A. solani* in unknown DNA samples (Fig. 3). When real-time PCR was conducted on *A. solani* pDNA and gDNA that was mixed with a tomato gDNA or DNA from other fungal isolates, no significant difference was observed between the resulting standard curves (P>0.05) or the correlation coefficients (R^2 =0.99), indicating no interactive effect of the plant or other DNA with the PCR reactions. The minimum detection of target DNA by real time PCR assay was 10fg of pDNA or gDNA, corresponding to Cp values of 26.28 and 28.35 respectively.

Melting curve analysis revealed that the melting temperatures of the PCR products and pDNA or gDNA of *A. solani* as template were uniform for all template concentrations, demonstrating the specificity of the amplification process (Fig. 4). Further, dissociation of the PCR reactions consistently produced a single peak at 74ºC in all the samples, showing the existence of single product in the reaction and no other primer dimer peaks were detected. Similar melting curve with a T_m maximum at 74.0°C was obtained also on DNA extracted from 10 conidia. To confirm the presence of a specific PCR product, a series of pDNA was analyzed after real-time PCR in 2% agarose gels. All the samples showed presence of expected band size of 537bp (Fig.5). Blastn analysis of 537 bp obtained from the sequencing of the amplicon showed 100% sequence identity with the ITS region of *A. solani* present in NCBI databases.

The conventional PCR was able to detect the pathogen 5 days post-inoculation in symptomatic leaves. However, real time PCR signals were detected in artificially inoculated tomato plants two days after inoculation, artificial inoculated seed and in naturally infected leaves, stems, calyx and fruits. No amplification was detected in the healthy plant material. A significant (P = 0.01) linear correlation was obtained between Cp values and log values of serial dilutions

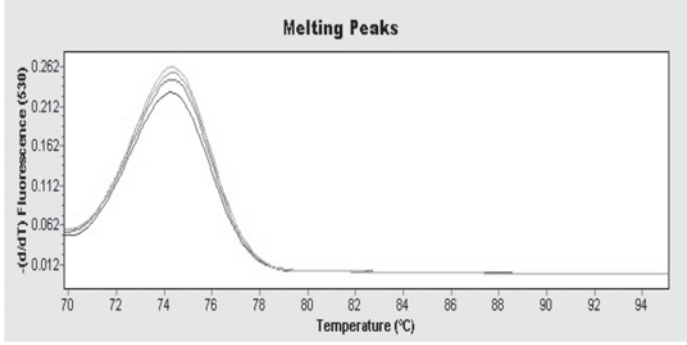
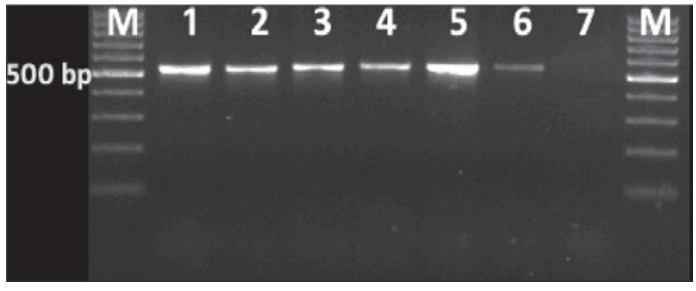


Fig.4. Melting curve profile for real-time PCR amplification. Four peaks represent target pDNA, target gDNA, target gDNA spiked with tomato DNA and target gDNA spiked DNA of *A. alternata*.



Lane M- 100 bp marker; Lane 1- artificially infected leaf; Lane 2- naturally infected leaf; Lane 3- naturally infected stem; Lane 4- naturally infected calyx; Lane 5- naturally infected fruit; Lane 6- infected seed; Lane 7- healthy leaf.

Fig. 5. *In Planta* detection of *A. solani* by real time PCR with primer ALP-F and ITS4

containing 1 to 0.001% infected leaf material (y= -3.50x + 30.59, R^2 = 0.9891) when serial dilution of artificially inoculated tomato leaves with healthy leaves were used to determine detection limit of *A. solani* in 100mg of tomato material. The infected leaf material with 0.001% infection had 156fg of DNA of *A. solani* for 100mg plant material while infected seed material with 0.1% had 85fg target of DNA for 25 seeds. Minimum 25 seeds are required to detect target DNA by real time PCR. Positive results were further confirmed by running the product of real-time PCR amplification on 1.5% agarose gel (Fig. 5). Real time PCR assay was able to detect *A. solani* in naturally infected leaves, stems, calyx, fruits and seeds collected from different localities in Karnataka (Fig. 5).

Alternaria solani is an important seed borne pathogenic fungus responsible for the early blight of tomato. Production and supply of disease free seed is necessary to limit the spread of this pathogen. Culture and morphological based detection methods are time consuming and laborious. In this study, ALP/ITS4 primers (Mckay *et al*, 1999) specifically detected *A. solani* in tomato seed by conventional and real-time PCR assay. Mckay *et al* (1999) reported that *A.linicola* and *A.solani* had identical ITS and ß–tubulin nucleotide sequences and prevented the selection of an *A.linicola*-specific PCR primer and suggested that this primer has the potential for use in detection of *A.solani* in many hosts. The results showed that ALP/ITS4 primers amplified DNA extracted from *A.solani* isolates.

A.solani is difficult to identify when conidia or other characteristic morphological structures are absent in the culture. In the absence of rapid morphological identification system, a technique that permits specific, sensitive and quantitative detection of *A.solani* in plant material is required. The present study describes a real time PCR based technique that meets these requirements that eliminate the need for pure culture isolation and production of morphological structures like conidia and shows the usefulness of the assay in artificially and naturally infected plant material. The real time assay developed in this study was highly sensitive with detection thresholds as low as 10fg gDNA and 10 conidia and 10 times more sensitive than that of conventional PCR. The lower detection limit by real-time PCR assays was possible due to less inhibition by plant inhibitors in real time PCR assays than that of conventional PCR. Real-time PCR assays have been used for the detection of fungal plant pathogens in seeds (Guillemette *et al*, 2004; Lievens *et al*, 2006; Alaei *et al*, 2009; Debode *et al*, 2009) and this technique would be useful for seed health testing. Thus, the results showed that real time PCR assay is a highly specific and sensitive technique that can be used in routine quarantine inspections to screen the seeds and transplants for the diagnosis of *A. solani*. The real-time PCR-based method presented here has the advantage of quantitative estimation of seed infection and can be automated easily over conventional PCR. This method can be used for diagnosis purpose in plant quarantine laboratory, disease screening programmes, epidemiological studies and for screening fungicidal resistance.

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