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ORIGINAL PAPER

DNA barcode, multiplex PCR and qPCR assay for diagnosis of pathogens infecting pulse crops to facilitate safe exchange and healthy conservation of germplasm

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Abstract
The DNA barcodes were developed from ITS region for the identification of fungal plant pathogens namely, *Alternaria alternata* and *A. tenuissima* both causing leaf spots, *Ascochyta rabiei* causing Ascochyta blight, *Fusarium oxysporum* f. sp. *ciceris* causing wilt, *Macrophomina phaseolina* causing dry root rot, *Rhizoctonia solani* causing web blight and wet root rot, *Sclerotium* (*Athelia) rolfsii* causing collar rot, *Sclerotinia sclerotiorum* causing stem rot and *Cercospora canescens* and *Pseudocercospora cruenta* both causing leaf spots in pulse crops. Barcode compliance for *A. alternata* (DBTPQ001-18), *A. tenuissima* (DBTPQ002-18), *A. rabiei* (DBTPQ003-18), *F. oxysporum* f. sp. *ciceris* (DBTPQ004-18), *M. phaseolina* (DBTPQ005-18), *R. solani* (DBTPQ006-18), *S. rolfsii* (DBTPQ007-18), *S. sclerotiorum* (DBTPQ008-18), *C. canescens* (DBTPQ009-18) and *P. cruenta* (DBTPQ029-20) have been generated based on the Barcode of Life Data System (BOLD) system. In addition to ITS, other genomic regions were also explored and on the basis of sequence variation they were ranked as TEF-α > SSU > LSU > β-tubulin. These genes could be considered for secondary barcode and phylogenetic relatedness. ITS-based markers for the detection of *A. alternata* (BAA2aF and BAA2aR) and *R. solani* (BRS17cF and BRS17cR) were developed which provided 400 bp and 220 bp amplicons, respectively. While, for *F. oxysporum* f. sp. *ciceris*, COX1-based marker (FOCox1F and FOCox3R) was developed which amplified 150 bp. The markers proved highly specific and sensitive with detection limit of 0.0001 ng of template DNA using qPCR and simultaneously detected these three pathogens. The DNA barcodes and diagnostics developed are suitable for quick and reliable detection of these pathogens during quarantine processing and field diagnostics.

Keywords Plant quarantine · Identification · Molecular detection · Fungal plant pathogens · Pulse crops

Introduction

Pulses are one of the major staple crops grown in major parts of the world including India, China, Canada, Myanmar, Nigeria, Brazil, Australia, USA, Russia, and Tanzania. Pulse production has increased considerably from 36 million MT during 1998 to 96.4 million MT in 2018 (FAOSTAT 2018). India has also shown an increased growth rate in pulse production during the last five years. In India, pulses are grown in 29.03 m ha area with 23.40 mt production and 806 kg ha^{-1} productivity (Anonymous 2020). Pulses are one of the most extensively consumed foods and are considered as a rich source of protein, fibre, vitamins, and minerals such as iron, zinc, and magnesium. Additionally, pulses have a unique nitrogen-fixing ability that contributes to soil fertility, thus avoiding usage of excessive chemical fertilizers in successive crops. Although, India is the largest producer of pulses (around 25% of global production), but the average yields are considerably lower than those of other major staple crops. Major factors that lead to such low yields are multiple abiotic as well as biotic constraints including several diseases and pests that impact pulse crop productivity and food security (Kakoti et al. 2020). Insects and diseases together are responsible for 5–10% yield loss in temperate regions and 50–100% in tropical region (Van Emden et al. 1988). In India, diseases caused 8–10% loss in pulse production annually (Vishwadhar and Chaudhary 2001). As per

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another estimate, annual yield loss in pulse crops due to diseases has been estimated to be up to 44% which depends largely on the crop variety being cultivated (Sandhya et al. 2017). Widespread occurrence of fungal diseases in pulse crops poses significant economic and social problems in India and other parts of the world (Sicard et al. 2018).

Seeds are the silent carriers, means of transmission and primary source of inoculum of plant pathogens. Both quantitative and qualitative losses are caused by seed-borne pathogens. Precise and timely diagnosis of a pathogen is a prerequisite for its effective management. An effective framework to contain the spread of pathogens via seed source or planting materials depends on the access to reliable, sensitive, specific, rapid, and robust identification techniques. Various traditional methods such as examination of dry and soaked seeds, incubation test on blotter, agar plate method, biochemical assays and microscopy are common and widely used. Morphology-based detections are skill-based, strenuous, time-consuming, stage-specific, and unable to resolve species ambiguities (Hoang et al. 2019). Also, all pathogenic fungi are unable to grow in standard laboratory conditions. Therefore, rapid and accurate sequence-based detection of fungal pathogens has now become widely popular (Luchi et al. 2020; Ren et al. 2020). A coherent novel technological, methodological approach such as species-specific molecular markers, quantitative real-time PCR assay (qRT-PCR) are needed for rapid molecular based detection of fungal pathogens (Owati et al. 2019).

The DNA barcoding is the most promising and beneficial technique for rapid identification and recognition of ambiguous and non-sporulating species across all fungal communities. Barcodes are universal, uniform, a short stretch of DNA usually 500–800 bp length, which are divergent at the species level and specific for a taxonomic group (Hoang et al. 2019). The internal transcribed spacer (ITS) region shows the highest probability of correct identification (PCI) for a large number of fungal pathogens with defined barcode gap (Schoch et al. 2012). Thus, the ITS regions have been recognized as the standard or primary barcode marker for fungi and possess an added advantage of easily amplifying most of the fungal taxa (White et al. 1990). Therefore, using the ITS marker alone for the identification is not sufficient in discriminating certain fungus clades. To address the limitations of the ITS-based primary barcode marker and to minimize this identification/barcode gap, a novel concept of additional barcode, popularly known as the secondary barcode was suggested by Stielow et al. (2015). Studies have shown that the TEF1-α gene is considered as one of the potential genetic markers for fungal DNA barcoding (Rehner and Buckley 2005). Other gene regions such as beta-tubulin (β-tubulin), largest subunit of RNA polymerase (RPB), calmodulin, cytochrome oxidase 1 (COX1), large subunit (LSU) and small subunit (SSU) are also very useful at species level identification for certain fungus lineages (Houbraken et al. 2011; Hustad and Miller 2015). SSU and LSU regions depict a varying level of genetic variation that enables the researchers to take up phylogenetic analysis at higher and intermediate taxonomic levels, respectively (Raja et al. 2017). The analytical performance of the detection technique tested by its specificity and sensitivity. The introduction of qRT-PCR allows the identification of target nucleotide with higher specificity and sensitivity (Luchi et al. 2020). This enhanced feature has increased its application in fungal identification taken directly from the symptomatic or asymptomatic plant parts.

The exchange of germplasm both at National and International levels has immense importance for their utilization in developing high yielding varieties. The germplasm exchange always has a risk of introduction of new exotic pathogens or newer virulent race/pathotype/biotype. Reliable identification and detection is essential to avoid introduction, spread and effective management of pathogens. DNA barcoding, PCR and qPCR-based techniques are both efficient and accurate for diagnostics of fungal plant pathogens. Thus, the present study was aimed to analyse the DNA barcode sequences using ITS 1 and ITS 2 universal primers and secondary barcode gene-specific markers such as TEF1-α, β-tubulin, COXI, LSU and SSU for 10 seed-borne fungal plant pathogens namely, *M. phaseolina*, *S. rolfsii*, *R. solani*, *S. sclerotiorum, A. rabiei*, *F. oxysporum* f. sp. *ciceris*, *A. alternata*, *A. tunuissima, P. cruenta* and *C. canescens* causing various diseases in pulse crops. Further, it was also aimed to develop and validate simplex and multiplex PCR and qPCR-based diagnostics for *R. solani, F. oxysporum* f. sp. *ciceris* and *A. alternata*.

Materials and methods

Isolation and maintenance of cultures of pathogenic fungi

Different fungal plant pathogens associated with the seeds of pulse crops namely, *A. alternata* (BAA2) and *A. tenuissima* (BAT3) both causing leaf spots, *A. rabiei* (BAR5) causing Ascochyta blight, *F. oxysporum* f. sp. *ciceris* (BFC7) causing wilt, *M. phaseolina* (BMP14) causing dry root rot, *R. solani* (BRS17) causing web blight and wet root rot, *S. (Athelia) rolfsii* (BSR19) causing collar rot, *S. sclerotiorum* (BSS21) causing stem rot, *C. canescens* (BCC23) and *P. cruenta* (BPG4) both causing leaf spots diseases were isolated using standard procedures of isolation from the infected seeds (Akhtar et al. 2014) and plant samples (Dubey and Singh 2008) of different pulse crops namely, chickpea, pigeonpea, mung/urdbean, French bean and cowpea. Some of the pathogens from other crops were also isolated for

comparison during the study (Table 1). These fungal cultures were purified and are being maintained on potato dextrose agar (PDA) slants. Identity of the various pathogens was confirmed through morphological features.

Extraction of DNA

DNA was extracted from the purified single spore cultures maintained for 2–3 weeks on shaking incubator (25 ± 1 °C; 120 rpm) on potato dextrose broth medium (20 g/l; Himedia) and further harvested using a paper filter. Liquid nitrogen was instantly added to the mycelia, which was then ground to a very fine powder and stored at − 80 °C. Modified CTAB method was used for total genomic DNA extraction (Murray and Thompson 1980; Dubey and Singh 2008). Purified DNA was subsequently dissolved in 200 µl TE buffer (pH 8, 10 mM Tris HCl and 1 mM sodium ethylene diamine tetraacetic acid) for further analysis.

Polymerase chain reactions (PCRs) of gene-specific primers

Amplification of six genes namely ITS, COI, β-tubulin, TEF-α, SSU, and LSU in different isolates of pathogens was performed using a set of universal primers (Table 2). PCR was performed using 1 × Taq polymerase buffer in 25 µl reaction mix containing approximately 25 ng of genomic DNA, 1 U Taq DNA polymerase, 0.6 mM of each dNTPs,1.5 mM of $MgCl_2$ and 10 pmol each PCR primer. The amplification was done with the PCR reaction conditions as described (Table 2). Agarose gel (1.2%) in 1 × TAE buffer was used to separate amplified PCR products by gel electrophoresis. Gel was stained with ethidium bromide and photographed using the UV-gel documentation system (G:Box, Syngene). Thereafter, the pathogen specific PCR amplified bands were gel eluted separately in different tubes. Subsequently, each of them was purified using gel extraction and purification kits (Qiagen India Pvt. Ltd.) in separate tubes. The purified samples were then sequenced by NGS method (Apical Scientific Sdn Bhd, Malaysia).

Barcode generation

The identity of the pathogens/sequences was confirmed using the NCBI-BLAST analysis tool (http://www.ncbi.nih.gov/index.html) showing 99–100% sequence similarity. Sequence trace files were assembled and consensus sequences constructed, aligned, and trimmed using BioEdit7.0.5 (Hall 1999). Specimen collection data, specimen images, consensus sequences and sequence trace files were uploaded to the Barcode of Life Data System (BOLD) and they are available for download as public project “Fungal pathogens of pulse crops (DBTPQ)”. Seventeen fungal plant pathogen isolates were utilized for DNA barcode analysis in the present study (Table 1). The edited sequences were submitted to the GenBank nucleotide sequence database and their respective accession numbers were obtained (Table 3).

Table 1 List of different fungal plant pathogens used in the present study

S. No.	Name of the fungal pathogen	Host	Working No.
1	*Alternaria alternata*	Wheat	BAA1
2	*A. alternata*	Mungbean	BAA2
3	*A. tenuissima*	Brassica	BAT3
4	*A. tenuissima*	Pigeonpea	BAT4
5	*Ascochyta rabiei*	Chickpea	BAR5
6	*A. rabiei*	Chickpea	BAR6
7	*Fusarium oxysporum* f. sp. *ciceris*	Chickpea	BFC7
8	*F. oxysporum* f. sp. *ciceris*	Chickpea	BFC8
9	*F. oxysporum* f. sp. *ciceris*	Chickpea	BFC9
10	*Macrophomina phaseolina*	Rice	BMP13
11	*M. phaseolina*	Chickpea	BMP14
12	*M. phaseolina*	Chickpea	BMP16
13	*Rhizoctonia solani*	Cowpea	BRS17
14	*Sclerotium rolfsii*	Chickpea	BSR19
15	*Sclerotinia sclerotiorum*	Chickpea	BSR21
16	*Cercospora canescens*	Mungbean	BCC23
17	*Pseudocercospora cruenta*	Cowpea	BPC24

Table 2 Gene specific PCR based primers for *ITS, COX, CAL, β-tubulin, TEF-α, SSU* and *LSU* genes, their sequences and amplicon size

Gene name	Primer sequence	PCR conditions	Amplicon size	Reference
ITS	ITS1: 5′-TCCGTAGGTGAACCTGCG G-3′ ITS4: 5′-TCCTCCGCTTATTGATAT GC-3′	Initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min; final elongation at 72 °C for 5 min	~600 bp	White et al. (1990)
COXI	COX1F: 5′-TTAGTGGGCCAGGAG TTC-3′ COX1R: 5′-ATACCACCAGCTAATACA G-3′	Initial denaturation at 94 °C for 5 min; 40 cycles of 94 °C for 1 min, annealing at 53 °C for 1 min and extension at 72 °C for 1 min; final elongation at 72 °C for 10 min	~500 bp	Damon et al. (2010)
β-tubulin	Bt1aF: 5′-TTCCCCCGTCTCCACTTC TTCATG-3′ Bt1bR: 5′-ACGAGATCGTTCATGTTG AACTC-3′	Initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C and extension at 72 °C for 1 min; final extension at 72 °C for 7 min	~600-bp	Jiménez-Gasco et al. (2002)
TEF-α	EF1-983F: 5′-GCYCCYGGHCAYCGT GAYTTYAT-3′ EF1-2218R: 5′-ATGACACCRACRGCR ACRGTYTG-3′	Touchdown PCR: initial denaturation at 94 °C for 2 min; 5 cycles of 94 °C for 30 s, annealing at 66 °C for 30 s and extension at 72 °C for 30 sec; 5 cycles of 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 sec; 20 cycles of 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min with final extension at 72 °C for 10 min	~1000 bp	Rehner and Buckley (2005)
SSU	NS1: 5′-GTAGTCATATGCTTGTCTC-3′ NS4: 5′-CTTCCGTCAATTCCTTTA AG-3′	Initial denaturation at 95 °C for 5 min; 30 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; final extension of 72 °C for 8 min	~1200 bp	White et al. (1990)
LSU	LROR: 5′-ACCCGCTGAACTTAAGC-3′ LR6: 5′-CGCCAGTTCTGCTTACC-3′	Initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 2 min; final elongation at 72 °C for 5 min	~1200 bp	Vilgalys and Hester (1990)

Phylogenetic analysis

The multiple sequence alignment (MSA) and pair-wise alignment were made using BioEdit version 7.0.5. The phylogenetic analysis utilizing the Maximum Likelihood (ML) method was performed in MEGA X software. The phylogenetic tree was constructed using CLUSTALW 1.8 sequence alignment tool and bootstrap neighbour-joining (NJ) method by MEGA X (64-bit for window) programme with 1000 bootstrap replicates.

Designing of species-specific markers and standardization of PCR conditions

Based on the sequence polymorphism of ITS and COI regions in different pathogens species, 12 species-specific primer sets were designed (Supplementary Table 1) for the detection of *R. solani, F. oxysporum* f. sp. *ciceris* and *A. alternata*. Standard primer designing criteria such as melting temperature, sequence specificity, no hairpin loop formation were taken into consideration. To determine melting temperatures and avoid any inter and intra primer interactions, the Multiple Primer Analyzer (MPA) available at http://www.thermoscientificbio.com/ web tools/multiple primer was used. Primers designed for different target species were able to amplify respective sample DNA of varying length so that the PCR products could be easily distinguished by their position in the gel. The primers specificity was confirmed by performing BLASTn in NCBI. The pathogen specific primers were also validated in the remaining species using conventional PCR. PCR was performed using 1 × Taq polymerase buffer in 25 µl reaction mix containing approximately 25 ng of genomic DNA, 1 U Taq DNA polymerase, 0.6 mM of each dNTPs, 1.5 mM of $MgCl_2$ and 5 pmol each PCR primer. The amplification was done with PCR reaction conditions as initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C