noxB-based marker for *Alternaria* spp.: a new diagnostic marker for specific and early detection in crop plants

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Abstract

Alternaria species are a major plant pathogen and their precise detection and identification is crucial for effective management. In the present study, a polymerase chain reaction (PCR)-based diagnostic technique has been developed for detection of *Alternaria* species. Four primers were designed for four genes viz. *noxB*, *AMK1*, *AKT3* and *NIK1*. In gradient PCR, only the primer sets for *noxB* gene showed specific amplicon of ~200 bp in all the isolates of *Alternaria*, while no amplification was observed in related fungal species such as *Ulocladium botrytis*, *Ulocladium consortiale*, *Stemphylium vesicarium*, *Cochliobolus tuberculatus*, *Curvularia prasadii*, and *Bipolaris sorokiniana*. The *noxB* primer set was used as diagnostic marker to discriminate and diagnose *Alternaria* species in nine different crop plants. Real-time assay revealed that the primer set was able to detect *Alternaria noxB* genes in leaves with no characteristic visible symptoms. Through real-time PCR, the *noxB* gene of *Alternaria* could be detected even in 0.5 ng of host DNA. This is the first report of *noxB* gene for molecular detection of *Alternaria* spp.

Keywords *Alternaria* · Diagnostic marker · Molecular detection · *noxB* · PCR · Real-time PCR

Introduction

The members of the genus *Alternaria* are cosmopolitan and known to be composed of 299 species (Nowicki et al. 2012; Wijayawardene et al. 2014). According to phylogenetic studies and sporulation patterns, the members of *Alternaria* genus have been clustered in several species-groups (Pryor and Gilbertson 2000; Chou and Wu 2002; Hong et al. 2005; Simmons 2007). The majority of them comprise of either saprophytic or plant pathogenic species of filamentous fungi, responsible for a number of crop diseases. The pathogenic members of this genus can cause losses in agriculture by reducing crop yield and through spoilage during storage (Barkai-Golan 2001; Ostry 2008). Few species are also capable of producing mycotoxins (King and Schade

1984; Woody and Chu 1992) which can infect plant products (Schroeder 1977) and are considered as a main cause of cancer (Blunden et al. 1991; Chu 1991; Liu et al. 1992; Pohland 1993).

The routine technique used for identification of pathogenic *Alternaria* spp. in the early phase of the infection is based on culture identification and quantification which involve a lot of expertise in morphology-based taxonomy, and is a tedious and time-consuming method (Lievens and Thomma 2005; Simmons 2007). Morphological identification becomes very difficult when some isolates under laboratory conditions produce sterile mycelium or sporulate sparsely (Guillemette et al. 2004). As *Alternaria* species is responsible for a wide range of diseases in major crop plants especially Solanaceous and Cruciferous crops, there is a need for a rapid and reliable method to detect the pathogen during early stage of infection, so that appropriate control strategies can be framed to prevent major economic losses. During last few decades, polymerase chain reaction (PCR)-based techniques have been used as a tool for the detection of various fungal pathogens which has replaced complex and time-consuming morphology-based identification (Schna et al. 2004). Nucleic acid-based methods are very robust and

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repeatable (Singh 2011). Such methods can be used at any developmental stage of plant since all living cells contain entire set of genome and not affected by environment. The polymerase chain reaction-based assays have long been used as tool for fungal detection (Ferrer et al. 2001). PCR-based methods for rapid and specific detection of *Alternaria solani* (Kumar et al. 2013), *Alternaria brassicicola* (Su'udi et al. 2013), *Alternaria alternata* (Ajiro et al. 2010; Zghair et al. 2014), *Alternaria helianthi* (Udayashankar et al. 2012) and *Alternaria dauci* (Benichou et al. 2009) have been reported. Further development of a rapid, reliable and specific detection of the pathogen using PCR-based diagnostic markers of small size and ease of amplification is quite useful for implementing remedial measures against the pathogen. Initially, internal transcribed spacers (ITS) had been the most preferred target for primer designing due to limited fungal genome data (Martin and Rygielwicz 2005). However, the copy number of such region is variable. Moreover, high-sequence conservation and presence in all fungal groups makes it difficult to design specific primers.

The aim of the study was to develop diagnostic marker using PCR assays to detect and identify *Alternaria* pathogens in diverse crop plants. Earlier, diagnostic markers using genes such as β -tubulin, cytochrome b, ITS1/ITS4, histone, PKSJ, and scytalone dehydratase 1 have been developed for different fungi. However, genes involved in pathogenesis are less explored, though they can be more useful for detection virulent species. The fungal NADPH oxidase (Nox) complex cassette codes for three enzymes viz. noxA, noxB and noxR (Yang and Chung 2013). noxB encoding NADPH oxidase enzyme is such a gene which has not been exploited earlier for molecular diagnostics of fungal pathogens. This enzyme is required in the ascospore germination and appressorium like structure formation that enables the fungus to penetrate the cuticle of the host plant (Egan et al. 2007; Morita et al. 2013). The study on fungal genomes identified up to four Nox genes in fungal species, but Nox genes are absent from available genomes of many hemiascomycete yeasts, unicellular Basidiomycetes and Zygomycetes, showed the variability in fungi. (Lalucque and Silar 2003; Takemoto et al. 2007). In the present study, we have developed noxB-based diagnostic marker capable of ecological monitoring of *Alternaria* infecting a wide-range crop.

Materials and methods

Fungal and bacterial isolates

Thirty-five isolates of *Alternaria* spp. belonging to different geographical regions collected from National Agriculturally Important Microbial Culture Collection (NAIMCC), ICAR-National Bureau of Agriculturally Important

Microorganisms (NBAIM), Mau, Uttar Pradesh, India were used in the present study (Table 1). The isolates represented eight different species of *Alternaria* such as *A. solani* (11), *A. alternata* (1), *A. brassicae* (11), *A. brassicicola* (3), *A. gossypina* (1), *A. tenuissima* (1), *A. porri* (4) and *A. sesame* (3). The fungal outgroups included in the study were *Ulocladium botrytis*, *Ulocladium consortiale*, *Stemphylium vesicarium*, *Cochliobolus tuberculatus*, *Curvularia prasadii*, *Bipolaris sorokiniana*, *Rhizoctonia solani*, *Fusarium oxysporum* and one bacterial isolate of *Bacillus subtilis* which were also obtained from NAIMCC. The fungal cultures were maintained on potato dextrose agar (PDA) medium at 28 ± 2 °C where as the bacterial culture was maintained on nutrient agar medium. The identity of the fungal isolates was validated through morphological and microscopic features.

Genomic DNA extraction

Fungal pure culture

Genomic DNA from fungal mass of 7-day-old culture was extracted following the method described by (Solanki et al. 2011; Kumar et al. 2013) with minor modifications.

Plant material

Both healthy and infected leaf tissues were taken from different plants like mustard and teak, whereas leaf samples with early infection symptoms in mustard (Fig. 1) and characteristic disease symptoms were collected from fields of Indian Institute of Seed Sciences (ICAR-IISS), Mau, Uttar Pradesh, India, while samples of cauliflower and brinjal were collected from farmer's field at Adari village, Mau, Uttar Pradesh, India. Okra, bottle gourd, sponge gourd, chilli, and cowpea samples were collected from different villages of Ghazipur district, Uttar Pradesh, India.

Genomic DNA from plant was extracted following standard methodologies (Doyle and Doyle 1987, 1990) with minor modifications. Both healthy and infected leaves were collected in the morning from field, washed and dried to remove surplus dirt. 0.5 g sample of both healthy and infected tissues was used for DNA extraction. Extracted DNA was suspended in 60 μ l of TE buffer and stored at -20 °C until further use.

Primer designing

Partial gene sequences were retrieved from PHI-base (<http://www.phi-base.org>) (Winnenburg 2006) having the Gene ID and Phi-BASE accession ID (Table 2). The DNA sequences were fed into Websat (Martins et al. 2009) online tool. This tool designs microsatellite-based markers which targets DNA flanked by microsatellites for the amplification

Table 1 Details of isolates of *Alternaria* species along with outgroups used in the present study

S. no.	Accession no.	Isolate(s)	Host	Geographical origin	Year of isolation
1.	NAIMCC-F-02600	<i>Alternaria brassicae</i> BAB-2	<i>Brassica juncea</i>	Jammu	2005
2.	NAIMCC-F-02601	<i>A. brassicae</i> BAB-3	<i>B. juncea</i>	West Bengal	2005
3.	NAIMCC-F-02606	<i>A. brassicae</i> BAB-18	<i>B. juncea</i>	Uttarakhand	2008
4.	NAIMCC-F-02608	<i>A. brassicae</i> BAB-29	<i>B. juncea</i>	Nagaland	2005
5.	NAIMCC-F-02610	<i>A. brassicae</i> BAB-39	<i>B. carinata</i> cv. javant	Himachal Pradesh	2010
6.	NAIMCC-F-02614	<i>A. brassicae</i> BAB-43	<i>B. juncea</i>	Jharkhand	2010
7.	NAIMCC-F-02615	<i>A. brassicae</i> BAB-44	<i>B. juncea</i>	Uttar Pradesh	2010
8.	NAIMCC-F-02617	<i>A. brassicae</i> BAB-47	<i>B. juncea</i>	Rajasthan	2010
9.	NAIMCC-F-02656	<i>A. solani</i> UPT-1	<i>Solanum lycopersicum</i>	Uttar Pradesh	2009
10.	NAIMCC-F-02659	<i>A. solani</i> JT-1	<i>S. lycopersicum</i>	Jammu and Kashmir	2010
11.	NAIMCC-F-02661	<i>A. solani</i> JT-3	<i>S. lycopersicum</i>	Jammu and Kashmir	2010
12.	NAIMCC-F-02662	<i>A. solani</i> JT-4	<i>S. lycopersicum</i>	Jammu and Kashmir	2010
13.	NAIMCC-F-02664	<i>A. solani</i> KT-6	<i>S. lycopersicum</i>	Himachal Pradesh	2010
14.	NAIMCC-F-02667	<i>A. solani</i> HPT-3	<i>S. lycopersicum</i>	Himachal Pradesh	2010
15.	NAIMCC-F-02668	<i>A. solani</i> HPT-4	<i>S. lycopersicum</i>	Himachal Pradesh	2010
16.	NAIMCC-F-02670	<i>A. solani</i> HPT-6	<i>S. lycopersicum</i>	Himachal Pradesh	2010
17.	NAIMCC-F-02672	<i>A. solani</i> OTA-38	<i>S. lycopersicum</i>	Karnataka	2011
18.	NAIMCC-F-02673	<i>A. solani</i> OTA-39	<i>S. lycopersicum</i>	Karnataka	2011
19.	NAIMCC-F-02678	<i>A. solani</i> OTA-66	<i>S. lycopersicum</i>	Karnataka	2011
20.	NAIMCC-F-00096	<i>A. brassicicola</i> No. 8	<i>Brassica juncea</i>	Karnataka	2008
21.	NAIMCC-F-02630	<i>A. brassicicola</i> OCA-14	<i>B. juncea</i>	India	2011
22.	NAIMCC-F-02633	<i>A. brassicicola</i> OCA-19	<i>B. juncea</i>	India	2011
23.	NAIMCC-F-00108	<i>A. porri</i> No. 4	<i>Allium cepa</i>	Karnataka	2008
24.	NAIMCC-F-02636	<i>A. porri</i> OOA-5	<i>A. cepa</i>	Karnataka	2011
25.	NAIMCC-F-02640	<i>A. porri</i> OOA-13	<i>A. cepa</i>	Karnataka	2011
26.	NAIMCC-F-00077	<i>A. alternata</i> No. 8	<i>S. lycopersicum</i>	Karnataka	2008
27.	NAIMCC-F-00098	<i>A. gossypina</i> CABI-329738	<i>Gossypium hirsutum</i>	Karnataka	1989
28.	NAIMCC-F-02648	<i>A. sesame</i> OSA 23	<i>S. indicum</i>	Karnataka	2011
29.	NAIMCC-F-00090	<i>A. brassicae</i> B-99	NA	New Delhi	2006
30.	NAIMCC- F-00104	<i>A. porri</i> No- 013	<i>Allium cepa</i>	Karnataka	2008
31.	NAIMCC- F-00132	<i>A. tenuissima</i> No-40	<i>S. lycopersicum</i>	Karnataka	2008
32.	NAIMCC- F-02621	<i>A. brassicae</i> OCA 3	NA	Karnataka	2011
33.	NAIMCC-F-02622	<i>A. brassicae</i> OCA 5	NA	Karnataka	2011
34.	NAIMCC-F-02649	<i>A. sesame</i> OSA 42	NA	Karnataka	2011
35.	NAIMCC- F-02653	<i>A. sesame</i> OSA 66	NA	Karnataka	2011
36.	NAIMCC-F-02305	<i>Ulocladium botrytis</i>	<i>S. tuberosum</i>	India	1978
37.	NAIMCC-F-01841	<i>Ulocladium consortiale</i>	Soil	India	1959
38.	NAIMCC-F-01681	<i>Stemphylium vesicarium</i>	<i>Allium cepa</i>	Pune	2008
39.	NAIMCC-F-00628	<i>Cochliobolus tuberculatus</i>	<i>P. drummondii</i>	Haryana	2008
40.	NAIMCC-F-0704	<i>Curvularia prasadii</i>	<i>S. tuberosum</i>	Rajasthan	1985
41.	NA	<i>Bipolaris sorokiniana</i>	<i>Hordeum vulgare</i>	Uttar Pradesh	2018
42.	NAIMCC-F-02974	<i>Rhizoctonia solani</i> RS 3	<i>O. sativa</i>	Uttar Pradesh	2011
43.	NA	<i>Fusarium oxysporum</i>	<i>Capsicum annum</i>	Jammu and Kashmir	2011
44.	NAIMCC-B-01495	<i>Bacillus subtilis</i>	Soil	Tamil Nadu	2014

NA not available

of the input gene sequences. The primers were checked for self-complementary and hairpin formation using OligoCalc (Kibbe 2007). These primers were further subjected to PCR using Primer Blast to check their specificity ([https://](https://www.ncbi.nlm.nih.gov)

www.ncbi.nlm.nih.gov). The primers were then synthesized (Eurofins India Pvt. Ltd.) and used for PCR assays.

Fig. 1 Mustard leaves with *Alternaria* infection in the initial stages

Table 2 Gene name, their Phi-BASE accession ID and Gene ID

Gene	Phi-BASE accession ID	Gene ID
NoxB	PHI:2938	AFP89626
AMK1	PHI:736	AAS201929
AKT3	PHI:2832	BAA07811
NIK1	PHI:1053	AY7000049

PCR assays

To determine the optimal annealing temperature of the designed primers, gradient PCR was used. The gradient

range used for different primers sets were determined based on the T_m value. The details of the gradient ranges for different sets of primers are presented in Table 3. PCR reactions were performed in a total volume of 25 μ l by mixing of 10 $\times$ PCR buffer (2.5 μ l), dNTPs (1.0 μ l), 0.5 μ l each of 10 μ M of forward and reverse primer, 0.4 μ l Taq DNA (1.0 U) polymerase (Bangalore Genei, India) and 50 ng of the template DNA. PCR was performed in thermal cycler (MyCycler Bio-Rad) with the PCR conditions: initial denaturation at 95 $^{\circ}$ C for 5 min followed by 35 cycles of denaturation at 95 $^{\circ}$ C for 1 min, annealing at 55.7 $^{\circ}$ C for 1 min, extension at 72 $^{\circ}$ C for 1 min and the final extension of 72 $^{\circ}$ C for 10 min. Amplicons were analyzed on 1.2% agarose gels containing ethidium bromide in TAE buffer and visualized under gel documentation system (Bio-Rad, Hercules, CA, USA).

The reliability and specificity was tested for the designed primers using: (1) DNA extracted from pure culture of fungus listed in Table 1; (2) DNA extracted from nine different crop species: (a) mustard, (b) cauliflower, (c) brinjal, (d) okra, (e) bottle gourd, (f) sponge gourd, (g) chilli, (h), teak, and (i) cowpea leaves infected with *Alternaria*; (3) DNA extracted from healthy leaves; and (4) DNA extracted from other unrelated fungi (i.e., *Ulocladium botrytis*, *Ulocladium consortiale*, *Stemphylium vesicarium*, *Cochliobolus tubercularatus*, *Curvularia prasadii*, *Bipolaris sorokiniana*, *Rhizoctonia solani* and *Fusarium oxysporum*) and bacterial isolate (*Bacillus subtilis*).

Sensitivity test and real-time detection of early infection

Sensitivity of the diagnostic marker was determined by qRT-PCR (G8830A AreaMx Real-Time PCR) using SYBR green as fluorescent molecules. DNA was extracted from samples having early infections in mustard (Fig. 1). The real-time PCR assay was performed using noxB primer set. DNA intensity and purity was quantified by Nano drop. DNA was serially diluted to 100 ng/ μ l, 10 ng/ μ l, 1.0 ng/ μ l, 0.5 ng/ μ l

Table 3 PCR primers designed in this study, their sequences and specificities

Primer set	Sequences	GC%	T_m ($^{\circ}$ C)	Mean T_m ($^{\circ}$ C)	Gradient temperature range ($^{\circ}$ C)
NoxB_F&R	CTTTGTGTTACCTTTGCGATA	40.91	57.52	57.85	55–61
	GACGGGCCATAGAAATTAGGTT	45.45	58.18		
AMK1_F&R	CGGTAGCTGTAGAAAGAT	44.4	51.4	54.8	52–57
	TCTGGGTCATGGTACGGC	61.1	58.2		
AKT3_F&R	CAGAGTGCTTTGCCACTTCTAC	50.00	60.3	60.3	57–63
	AGGCAGTTAGTTGTAGCTTCGG	50.00	60.3		
NIK1_1F&R	GTACGTTCCCTTGTCGCTAGTT	50.00	60.3	58.4	52–62
	GTAATTCGATAGCCTGCCAAAA	40.9	56.5		

and 0.1 ng/μl using nuclease-free MiliQ water. All dilutions of DNA (10–0.1 ng/μl) were used as template for performing the qRT-PCR experiments. Each 20 μl of PCR reaction mixture contained 10 μl 2×SYBR green Master mix, 0.5 μl QN ROX Reference Dye, 1 μl each of primer F and primer R, respectively (10 pmol), 1 μl DNA and 6.5 μl of nuclease-free water. qRT-PCR conditions were: initial heat activation

at 95 °C for 10 min, followed by 40 cycles of amplification by 3-step cycling, denaturation at 95 °C for 5 s, annealing at 55.7 °C for 30 s, extension at 72 °C for 15 s. Melting curve analysis was performed by heating the plate at 95 °C for 30 s, and incubating at 65 °C for 30 s, then heating to 95 °C for 30 s. Experiment was carried out in triplicates. Fluorescent signal intensities were recorded during the melting experiment. Result was analyzed by the Agilent AreaMx

Table 4 List of trimmed sequences of 21 PCR-purified amplicons of *noxB* gene from different sp. of *Alternaria*

S. no.	Accession no.	Isolate(s)	Size of trimmed sequence used for blast (bp)	% Similarity	Closest match
1	NAIMCC-F-02600	<i>Alternaria brassicae</i> BAB-2	151	98	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds, JX136700.1
2	NAIMCC-F-02606	<i>A. brassicae</i> BAB-18	152	99	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
3	NAIMCC-F-02614	<i>A. brassicae</i> BAB-43	145	100	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
4	NAIMCC-F-02617	<i>A. brassicae</i> BAB-47	139	100	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
5	NAIMCC-F-02656	<i>A. solani</i> UPT-1	143	100	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
6	NAIMCC-F-02661	<i>A. solani</i> JT-3	133	100	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
7	NAIMCC-F-02668	<i>A. solani</i> HPT-4	149	100	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
8	NAIMCC-F-02672	<i>A. solani</i> OTA-38	151	98	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
9	NAIMCC-F-02678	<i>A. solani</i> OTA-66	152	98	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
10	NAIMCC-F-00096	<i>A. brassicicola</i> no. 8	139	100	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
11	NAIMCC-F-02630	<i>A. brassicicola</i> OCA-14	148	97	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
12	NAIMCC-F-02633	<i>A. brassicicola</i> OCA-19	153	98	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
13	NAIMCC-F-00098	<i>A. gossypina</i>	137	100	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
14	NAIMCC-F-00077	<i>A. alternata</i> no. 8	153	98	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
15	NAIMCC-F-02648	<i>A. sesame</i> OSA 23	137	100	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
16	NAIMCC-F-02670	<i>A. solani</i> HPT-6	147	97	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
17	NAIMCC-F-02672	<i>A. solani</i> OTA-38	147	97	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
18	NAIMCC-F-02673	<i>A. solani</i> OTA-39	144	98	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
19	NAIMCC-F-02678	<i>A. solani</i> OTA-66	142	100	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
20	NAIMCC-F-00096	<i>A. brassicicola</i> no. 8	147	99	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1
21	NAIMCC-F-02630	<i>A. brassicicola</i> OCA-14	140	100	<i>Alternaria alternata</i> strain EVMIL 31 NADPH oxidase B (NoxB) gene, complete cds JX136700.1

Fig. 2 PCR amplification using primer pair NoxB_1F and NoxB_1R showing amplification product of ~203 bp. Lane M is a 100-bp DNA marker. Lanes 1–35 represent *Alternaria* spp.; while lanes 36–44 are *Ulocladium botrytis*, *Ulocladium consortiale*, *Stemphylium vesicarium*, *Cochliobolus tuberculatus*, *Curvularia prasadii*, *Bipolaris sorokiniana*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Bacillus subtilis*

software version 1.5. A standard curve (fluorescence vs cycles) was plotted using the obtained data.

Sequencing of *NoxB* amplicons

Twenty-one PCR-purified amplicons of *noxB* gene from different sp. of *Alternaria* were sequenced using ABI3780 XL sequencer (Sanger sequencing) at SciGenome Labs. Trimmed sequences provided by SciGenome Labs were used for further analysis (Table 4).

Results

Primer design and specificity of PCR

A total of four primers were initially designed for four different genes viz. *noxB*, *AMK1*, *AKT3* and *NIK1*. All the primers showed specific amplification during primer blast search (Table 3). When these primers were subjected to gradient PCR, only the primer sets for *noxB* gene showed specific amplification. The *noxB*-specific primers generated a specific amplicon of ~200 bp in all the isolates of *Alternaria*, while no amplification was observed in fungal

(*Ulocladium*, *Stemphylium*, *Cochliobolus*, *Bipolaris*, *Curvularia*, and *Rhizoctonia*) and bacterial (*Bacillus*) out groups (Fig. 2). When the *noxB* gene sequences were subjected to NCBI BLAST search, all the sequences showed (97–100)% similarity with *noxB* gene (JX136700.1) of *Alternaria alternata* strain.

Detection of *Alternaria* pathogen in infected leaves

When the *noxB* primer set was tested on diseased samples of nine host crops viz. mustard, cauliflower, brinjal, okra, bottle gourd, sponge gourd, chilli, teak, and cowpea, it could clearly differentiate between healthy and *Alternaria*-infected samples with specific amplification (~200 bp) in infected samples (Fig. 3). In case of mustard when the primer set was tested on plant samples with early symptoms, it could clearly detect the presence of *Alternaria* through amplification of the ~200 bp fragment. Further, *Alternaria* sp. was recorded from all the diseased plant samples and their identity was confirmed through morphological and microscopic studies. Three fungal isolates obtained from the plants viz. mustard, brinjal and cauliflower had distinctly different colors of colony while all of them had muriform, brown conidia characteristic to *Alternaria*.

Fig. 3 Infected and healthy leaves of nine different crops are shown in the diagram. Red arrows mark the infected portions of the leaves along with the PCR-based detection using NoxB primers of the

respective crops viz. mustard, cauliflower, brinjal, chilli, bottle gourd, sponge gourd, teak, okra and cowpea

Sensitivity and early detection using qRT-PCR

When the DNA extracted from leaves with early symptoms (tiny brown spots without any characteristic concentric rings) indicating probable *Alternaria* infection was subjected to real-time PCR using NoxB primer set, amplification was observed till 0.5 ng/μl (Fig. 4). No amplification was observed in 0.1 ng/μl.

Discussion

Accurate and rapid detection of pathogens is important for effective management of plant pathogens. However, detection of phytopathogenic fungi in plant materials, especially at low infection levels is quite difficult, though it can help

to take protective measures to significantly reduce the crop loss. In this study, we have developed a *noxB* gene-based diagnostic marker for detection of *Alternaria* spp. *noxB* gene has been reported to be present in a wide range of fungi especially Ascomycetes and Basidiomycetes, however, has been reported to be absent in Saccharomycotina, Taphrinomycotina and Ustilaginomycotina (Takemoto et al. 2007; Woudenberg et al. 2013). However, there is no published report till date for using *noxB* gene as diagnostic primer for any fungi. This study is the first report of using *noxB* gene as a diagnostic primer for detection of *Alternaria* spp. PCR-based detection of pure cultures of *Alternaria* isolates showed positive amplification while no amplification was obtained for other fungi or bacteria indicating its specificity for *Alternaria*. Sequencing of the *noxB* amplicons and consequent BLAST search further supported the detection

Fig. 4 Standard curve for absolute quantification of genomic DNA generated with tenfold serial dilutions of genomic DNA isolated from infected plant material of probable *Alternaria* infection using *noxB* primer sets. The curves show the relative fluorescence intensity with respect to the number of PCR cycles

of *Alternaria* pure cultures. Further, the designed primer sets could clearly distinguish between healthy and *Alternaria*-infected leaf samples and even detect the presence of pathogen in samples with the early symptoms. Real-time analysis revealed that the *noxB* primer set was capable of detecting the *Alternaria* infection before expression of robust visible symptoms. It could also detect the *noxB* genes in 0.5 ng/ μ l of DNA extracted from infected leaves. Such markers can be very useful for ecological monitoring of plant pathogens and as their use can help to decide appropriate management strategy before much loss has been caused by pathogen. The conventional methodologies have relied either upon on field identification of disease with the presence of characteristic symptoms, culturing the pathogen from diseased plant parts or visual identification and counting of pathogen propagules. As such approach is largely based on taxonomic expertise and involves a significant time, it may not help to design control strategies in advance. Therefore, specific molecular markers with the ability to detect the pathogens at early stages will be very useful to reduce economic losses. Earlier, Kumar et al. (2013) have also developed β tubulin-based diagnostic primers for quantitative detection of *Alternaria solani* in tomato. Su'udi et al. (2013) also developed real-time quantification method for detection of *Alternaria brassicicola* using Scytalone dehydratase 1 gene. However, these studies report purely lab-based detection of pathogens where the plants were artificially inoculated. In the present report, we have tested the primer set with the leaf samples collected from farmer's field where chances of mixed infection remained in a high proportion. Moreover, the sensitivity of the primer set was also high enough to detect the infection. The generic specificity of the primer sets gives it a wider applicability as evident from its detection of pathogen from nine different crop plants. To the best of our knowledge, this is the first report of *noxB* gene-based molecular diagnostic method for detection of *Alternaria* spp.

In conclusion, the *noxB* gene-based PCR assay can be very useful for specific detection of diverse *Alternaria* spp. from a diverse range of host and can help in rational selection of fungicides to manage leaf lesions and blights diseases in different crops. Especially, its utility in early stages of disease development will definitely help to devise control strategies for reducing economic losses.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest. All the authors contributed equally in this work.

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