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International Journal of Current Research
Vol. 6, Issue, 09, pp.8335-8350, September, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

MOLECULAR AND PATHOGENIC DIVERSITY OF *ALTERNARIA* SP. ISOLATED FROM SESAMUM BY SCAR MARKER

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ARTICLE INFO

Article History:
Received 05th June, 2014
Received in revised form
23rd July, 2014
Accepted 20th August, 2014
Published online 18th September, 2014

Key words:

Diversity,
PCR,
RAPD,
Sesame,
Pathogenicity,
SCAR marker.

ABSTRACT

A total of 164 *Alternaria* isolates were obtained by following standard tissue isolation method on potato dextrose agar media plates. Based on morphological features *viz*., growth, sporulation pattern, 45 isolates were identified as *A. alternata* and 28 were *A. sesami* and the remaining ones were non-sporulating. The colour of the isolates ranged from grey to light brown and both fluffy (37) and smooth type (30) of growth were observed with regular to irregular margin. Majority of the isolates were fast growing (119) and some were moderate (45) in growth. Twenty five percent of the isolates (40 isolates) were highly sporulating and some of them (54) were shy in sporulation. Individual isolates were studied in detail on type of growth and margin, color of the colony, radial growth, sporulation, width of the mycelium, vertical (0-3) and horizontal (2-5) septation of conidia, size of conidia (33.1-196 x 24.4-78.6 µm) and length of beak (7.1-88 µm). Out of 164 isolates, twenty one isolates were highly virulent mostly dominated by *A. alternata* and other species, nine of them were moderately virulent and fourteen were less virulent. Based on ITS region, *Alternaria* spp were identified and found 8 different species and among all *A. alternata* was found dominant. RAPD was also studied using different RAPD primers and found that all the species formed similar clusters with one another in their homology. Among them, *A. sesami* produced a single specific band with OPM-4 primer and *A. sesami* species specific band has been identified. *A. sesami* specific gene has been identified and further cloning, sequencing of the +ve cloned cells revealed that they formed 25 sets of primers. Among them, 10 were synthesized and sequence characterized amplified region (SCAR) marker of the *A. sesami* has been developed.

INTRODUCTION

Alternaria leaf blight is one of the most common fungal diseases of major food crops. *Alternaria* affects both leaves and fruits, on foliage the development of black large necrotic lesions in the center and lesions are generally due to the abundant production of fungal spores and on fruits small yellow halo necrotic spots are seen. In recent years, *Alternaria* blight has gained utmost significance as a foliar disease on many crops such as sesame, sunflower, tomato, mustard, rape-seed, vegetable etc (Guleria and Kumar, 2006; Savitha *et al.,* 2012). *A. alternata* is the causal agent of major oil seed crops including sesame and there is a considerable range of variation in conidial morphology in regard to size, shape, septation, color, and ornamentation that is dependent upon conidial age (Berry, 1960; Ostry, 2008; Ramjagathesh and Ebenezer, 2012). It is an important annual crop in the tropics and subtropics; sesame oil has good shelf life because of the presence of endogenous antioxidants, sesamin, sesamol and sesaminol together with tocophenols, while its protein is used for industrial purposes (Lubiana and Murugan, 2012). *A. alternata* is ubiquitous and it is known to cause several crop diseases further complicating the taxonomy of this group of fungi in the presence of numerous isolates with intermediate characteristics that do not clearly segregate into recognized species (Woody and Chu, 1992). Thus, differentiating these fungi can be difficult for those not familiar with the specific morphological characteristics that separate these species and it has been suggested that these fungi, in particular *A. alternata*, are frequently misidentified (Roberts *et al*., 2000; Shindhe *et al*., 2011). In addition to morphology, variation exists among small spored catenulate *Alternaria* taxa in respect to host range. This approach has given rise to several newly described *Alternaria* spp. (Tiwari *et al*., 2011), or a reclassification of formerly described taxa, such as *A. arborescens*, formerly known as *A. alternata* f. sp. *lycopersici* (White *et al*., 1990). Considering the diversity of conidial shapes and sizes among *Alternaria* spp. in general, there have been efforts in developing sub-generic groupings of species based upon similar conidial characteristics. Analyses of DNA-DNA reassociation kinetics revealed high DNA homology

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among several small spored host-specific *Alternaria* spp. and *A. alternata*, which suggests these species represent intra specific taxa (Guleria and Kumar, 2006). Supporting these results, restriction fragment length polymorphism (RFLP) analyses using the λ phage clone Alt1 as a hybridization probe were not able to differentiate small-spore host-specific *Alternaria* spp. from *A. alternata* (Guo *et al*., 2004). *Alternaria* toxins have received increasing attention both in research as well as risk assessment studies. The production of varied kind of toxins by *Alternaria* spp. including host specific toxins has thrown up further challenges for research. However, random amplified polymorphism DNA (RAPD) analyses of *A. alternata, A. tenuissima, A. infectoria*, and small spored host specific species revealed distinctive RAPD fragment patterns for all species, and cluster analysis did resolve these species into distinct clades, which suggests that these taxa constitute well defined species (Roberts *et al*., 2000). In summary, previous molecular studies have not produced a consensus on the genetic relationships among small spored catenulate *Alternaria* spp. which underscores the controversial taxonomic status of this group of fungi. The purpose of this work was to examine morphological diversity among *Alternaria* isolates from sesame and to determine the temporal and spatial distribution of this diversity. An additional objective of this investigation was taken up to evaluate the phylogenetic relationships among *Alternaria* isolates from sesame and representative isolates from the various small spored catenulate species groups using a variety of molecular techniques in an effort to further our understanding of the taxonomic status of these *Alternaria* spp.

MATERIALS AND METHODS

Collection and isolation of *Alternaria* species

The sesame leaf infected with *Alternaria* showing typical blight symptoms samples were collected from major sesame growing states of India *viz*., Andhra Pradesh, Bihar, Delhi, Karnataka, Madhya Pradesh, Maharashtra, Rajasthan, Uttar Pradesh and Tamil Nadu. The *Alternaria* strains were isolated by standard tissue isolation technique from *Alternaria* blight infected leaf of sesame under aseptic condition (Bhaskaran and Kandaswamy, 1978). The infected leaf tissues were cut into small bits of 1-2 mm size and surface sterilized in 1:1000 mercuric chloride ($HgCl_2$) solution for 1 min and washed repeatedly in sterile distilled water to remove the traces of mercuric chloride. The small cut leaf pieces were placed on to a Petri plates containing potato dextrose agar (PDA) under aseptic conditions (Savitha *et al*., 2012). All the inoculated plates were incubated at 28±2º C for 7 days. The pure culture of the fungus was obtained by single spore isolation method.

Cultural and morphological characterization of *Alternaria* isolates

All the isolates were screened based on the morphological characters on the PDA plates. The parameters used for cultural studies are the types of colony, color of the colony, growth of margins, color of margin, mean radial growth and sporulation etc. Morphological characters such as mycelial width, length and width of conidia, number of horizontal and vertical septa and length of beak which were measured at 10x magnifications microscope. The different spore patterns were studied to differentiate the organism (Meena *et al*., 2012; Savitha *et al*., 2012). The isolates were maintained in slants for further characterization purposes. The pure culture was subcultured on PDA slants and kept at 28±2º C for 9 days.

Pathogenicity tests

The representative isolates were chosen for virulence test based on growth, sporulation and representation from different geographical regions. The fast growing, highly sporulating and representative isolates from different geographical regions were selected for virulence test on popularly grown sesame E8 cultivar. The leaves of sesame plants were inoculated with spore and mycelial suspension ($1x10^6$ spores/ ml) of the fungus by using atomizer in a moist chamber. After 48 hr of inoculation, the observations were made for symptom development and suitable control was maintained by spraying with distilled water (Amaresh and Nargund, 2005).

DNA extraction

The DNA was extracted from the 7 days old *Alternaria* fungal mycelium by CTAB method collected from potato dextrose broth and filtered through pre-autoclaved filter paper. The mycelia were then dried completely and grounded with liquid nitrogen using pestle and mortar. The grinded material were transferred to 1.5 ml Eppendorf tubes and mixed gently by inversion. 500 µl of extraction buffer (CTAB powder + CTAB buffer + mercaptaethanol) and Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution was added to tubes and the vials were incubated at 55^o-60º C for 20 min in water bath. The samples were centrifuged at 10000 rpm at 4º C for 1hr, the aqueous layer was transferred to a fresh vials and 2.0 µl RNase A (20mg ml^{-1}) were added and mixed gently by inverting and incubated for 37º C for 10min. An equal volume of chloroform: isoamylalcohol (24:1) was added and centrifuged at 10000 rpm for 15-20 min; upper aqueous layer was collected in fresh vials. A $3/4^{th}$ volume of ice cold isopropanol was added to each vial and mixed gently till white fluffy DNA precipitate appears. The vials were centrifuged at 12000 rpm for 5 min to pellet the DNA, the supernatant was discarded and 70% of cold ethanol (100µl) was added to the vials. The vials were centrifuged 10000 rpm for 3-5 min and flow through liquid ethanol was discarded and the vials were dried for an hour to remove the excess ethanol and 30 µl of elution buffer was added to the vials and allowed to dissolve the DNA pellet (White *et al*., 1990; McKay *et al*., 1999; Savitha *et al*., 2012).

PCR amplification

The purified DNA was amplified with universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS–4 (5'-TCCTCCGCTTATTGATATGC-3'). The 25µl PCR reaction mixtures contains DNA template 50ng, 1X *Taq* buffer, 0.2mM of each of deoxyribonucleotide triphosphate mixture, 1µM of each primer, 1.5mM $MgCl_2$, 2 U of *Taq* DNA polymerase (Bangalore Genei). The PCR conditions were, one cycle of initial denaturation (94º C for 4 min), 36 cycles of denaturation (94 º C for 1 min), annnealing (55 º C for 1min), extension (72

º C for 1.5 min) and one cycle of final extension (72 º C for 5 min) (White *et al*., 1990; Yu *et al*., 1982; McKay *et al*., 1999). The amplified product was checked in 1.5 % agarose gel for further confirmation.

Cloning, sequencing and phylogenetic analysis

PCR amplified products were cloned into pGEM-T Easy Vector Systems (Promega) and transformed into competent *E. coli* strain DH5α by following manufacturer's instructions. Plasmid DNA was isolated and the presence of the insert was confirmed by restriction digestion of plasmid DNA with *Eco*RI restriction enzymes (Fermentas Life Sciences, Canada). Clones of interest were sequenced (GeNei, Bengaluru, India) by using M-13 sequencing primer and the sequences were confirmed with NCBI BLAST database for the identity. The sequences were confirmed with NCBI BLAST database for the identity of the isolates based on previously published sequences and were used for phylogenetic analyses (Chennappa *et al*., 2013). All the *Alternaria* strain sequences were deposited in NCBI genbank along with location of the isolates.

Screening of RAPD (Randomized Amplified Polymorphic DNA) primers

A total of 25 primers were used for screening and out of 25 primers (OPA, OPD, OPM, OPG, OPT and OPV) 19 primers were selected for RAPD analyses. All the 164 *Alternaria* isolates were carried out for RAPD analyses with a series of the primers and the diversity of the each isolates was obtained with different polymorphism.

PCR for RAPD

A total of 19 random primers were (Operon Technologies) used such as OPA01, OPA02, OPA09, OPA13, OPA16, OPA17, OPD01, OPD02, OPD03, OPD04, OPD05, OPD07, OPD08, OPD11, OPD12, OPD13, OPD14, OPD15 and OPD20. The DNA was amplified using RAPD technique used to produce amplified fragments ranging from 200±900 bp. Each isolate was tested at a range of DNA concentrations and the highest concentration that resulted in the clearest amplification of RAPD bands was used. This corresponded to 0±5 to 2±5ng DNA for all isolates in a reaction volume of 25µl. In addition, each reaction contained 400 nm 10 bp primers, 1±5 mm $MgCl_2$ and 3±5 U Taq polymerase. DNA was amplified in a thermal cycler with the following programme: 94 º C for 1 min, initial denaturation cycle ; 1 min at 94° C, 1 min at 35° C, 2 min at 72° C, 37 cycles ; 7 min at 72° C, 1 cycle (Khodadadi *et al*., 2011). The PCR products were separated on 2% agarose gel and banding patterns were documented in Syngene gel documentation unit.

RAPD data analysis

Only bright and clearly visible bands were selected and scored. Statistical analysis of the data was performed using the NTSYS-pc program. The degree of genetic relatedness or similarity was estimated using the Jaccard coefficient [*a*}(*n*±*d*)] in which the data are defined by a two-way contingency table such that for any pair wise comparison of isolates, *a* (1, 1), *b* (1, 0), *c* (0, 1), *d* (0, 0), with ` 1' denoting presence of a band and ` 0' denoting absence of a band, and *n* (*a*-*b*-*c*-*d*). Clustering of similarity matrices was by UPGMA and the projection of phenograms was done using the TREE program of NTSYS-pc. A Q-type PCA was also performed using NTSYS-pc as an independent test of the clustering of isolates by UPGMA (Guo *et al*., 2004; Morris *et al*., 2000).

SCAR Marker development for *Alternaria sesame*

The aim of the present experiment was to tag the candidate genes, responsible for imparting resistance/ susceptibility to *Alternaria* blight which is among the major biotic stresses, which results in retarding the yield, oil-quality and economic losses.

Screening and selection of RAPD primers

A total of 25 primers were used for screening and out of 25 primers OPA, OPD, OPM, OPG, OPT and OPV primers were selected for SCAR marked detection for the species specific bands generated distinctly unique bands in causing *Alternaria* blight disease in sesame. The band length of the species is around 750 bps and all the species have produced some specific bands in the gel after amplification with theses primers.

Identification of specific bands and gel extraction

Based on the species specific bands generated in the gel picture bands were selected and were eluted from the gels. The eluted bands were further purified and amplified with once again for reproducibility check purpose and after confirming the band the PCR amplicons were purified and used for cloning purposes using Axygen PCR purification kit.

Preparation of cloning vectors and competent cells

The DNA stock obtained was quantified using Nanodrop and working concentrations of 20ng/µL and 40ng/µL were prepared for each sample. Dilutions of DNA from all the species of *Alternaria* were separated and RAPD primers obtained from OPERON Technologies (OPA – OPX) were used to screen the bulks for polymorphism. Chemically competent *E. coli* cells were prepared and transformed by KCM method. DH5α cultures were grown in Luria-Bertani (LB) to OD_{600} of 0.4-0.5 by using fresh cultures (Promega Cloning and Vector Ligation Kit).

Transformation with KCM competent cells

After the growth of the cells in LB medium the cells were allowed to thaw an aliquot on ice. The second set up tube containing 20 µl of 5X KCM were prepared and DNA was added to the ddH_20 up to a total volume of 100 µl. 100 µl of the cell suspension was added to the second tube mixed and kept it on ice for 20 minutes. The cells were heat shocked by keeping to 37°C for 5 minutes. 1 ml of pre-warmed LB or SOC medium was added to the tube and incubated at 37°C for 40-60 min. 100 µl cells content was transformed onto LB-ampicillin

plate. Spin down the remaining cells, re-suspended into 100 µl of fresh LB and plate onto a second plate.

DNA Ligation

The purified product was ligated onto a pGEM-T Easy cloning vector. The ligated product was used to tranform chemically competent cells and ligation was carried using pGEM-T Easy cloning kit. Typical reaction conditions for directional cloning ligation: 20 ng of vector, 3-fold molar excess of insert, 2 µL of ligase, 20 µL reaction volume and Ligated at 15° C for 2 hours to overnight.

Bacterial transformation

Highly competent cells were used for blunt ended cloning or very large constructs. Only ½ of the DNA transformed into bacteria and incubated in 1 mL of LB for 30-60 min. (the remaining 1/2 of the ligation mixture to run on a gel to check the ligation competence of fragments). Blue/White colony cells were selected and evenly spread 120 µL X-gal/IPTG across plates > 1 hour before for use. 100 µL of bacteria at different dilutions in LB: 1/100, 1/10, 1x, and 10x (for the latter spin 1 mL of culture were plated and resuspend in 0.1 mL LB). The DNA minipreps were prepared using Axygen preparation kit. The sequencing results were screened for removing vector regions.

Sequencing of positive clones

After cloning of insert, the three positive cloned colonies were sequenced and the based on the insert and the sequence data primers were synthesized for validation of the insert or the required specific primer.

Nucleotide sequence accession number

All the sequences of *Alternaria* isolates were deposited in NCBI genbank along with location of the isolates. Accession numbers are: KF193431, KF193432, KF193433, KF193434, KF193435, KF193436, KF193437, KF193438, KF193439, KF193440, KF193441, KF193442, KF193443, KF193444, KF193445, KF193446, KF193447, KF193448, KF193449, KF193450, KF193451, KF193452, KF193453, KF193454, KF193455, KF193456, KF193457, KF193458, KF193459, KF193460, KF193461, KF193462, KF193463, KF193464, KF193465, KF193466, KF193467, KF193468, KF193469, KF193470, KF193471, KF193472, KF193473, KF193474, KF193475, KF193476, KF193477, KF193478, KF193479, KF193480, KF193481, KF193482, KF193483, KF193484, KF193485, KF193486, KF193487, KF193488, KF193489, KF193490, KF193491, KF193492, KF193493, KF193494, KF193495, KF193496, KF193497, KF193498, KF193499, KF193500, KF193501, KF193502, KF193503, KF193504, KF193505, KF193506, KF193507, KF193508, KF193509, KF193510, KF193511, KF193512, KF193513, KF193514, KF193515, KF193516 and KF193517

RESULTS AND DISCUSSION

Isolation of *Alternaria* species

Survey was conducted to know the incidence, severity of *Alternaria* blight of sesame and concentric zoned blight symptoms samples were collected from nine states of India (Fig 1). The standard tissue isolation technique was followed for the isolation of *Alternaria* under aseptic condition. After 7 of days incubation, brown to black colored colonies of *Alternaria* cultures were observed and pure culture of the fungus was obtained by single spore isolation method. *Alternaria* is a ubiquitous and includes saprophytic, endophytic and pathogenic species. The cosmopolitan nature of small spored *Alternaria* species makes them important in a broad range of disciplines. As saprophytic, they can spoil food products and animal feedstuffs by deterioration and by production of biological active compounds (Ostry, 2008). Rao and Vijayalaxmi, (2000) reported the occurrence and distribution of *Alternaria* species causing blight in sesame and phytotoxicity that the *Alternaria* toxin can cause blight and responsible for the appearance of the symptoms. Similar kind of disease symptoms were observed on plant of sesame as concentric rings on the leaves, which turns brown with grey centre. As the disease advances, the spots become oval or circular and become irregular in shape. The infected leaves become dry and lead to the defoliation. Rajpurohit, (2011) reported similar kind of morphological characters in Niger due to blight of *Alternaria porri* and *A. alternata*. *Alternaria* infected leaves developed brown, necrotic lesions with or without chlorotic bands and with or without concentric rings (Mmbaga *et al*., 2011).

Cultural and morphological characterization

Isolates were characterized for morphological and cultural diversity. Culturally a good amount of variations was observed during the growth. As many as 164 isolates of *Alternaria* were isolated for the morphological and cultural diversity. Among them, 65 isolates were identified as *A. alternata* and 52 were *A. sesami* based on morphological features. The remaining ones were non-sporulating. The color of the isolates ranged from grey to light brown, both fluffy type (37) and smooth type (30) of growth were observed with regular (115) to irregular margin (49 isolates). Majority of the isolates were fast growing (119) and some were moderate (43) in growth (Fig 2 and Table 1). Twenty five Percent of the isolates (40) were highly sporulating and some of them (54) were shy in sporulation and other isolates were non sporulating (50). Individual isolates showed varied type of growth, margin, color, radial growth, sporulation, width of the mycelium, vertical (0-3µm) and horizontal (2-5µm) septation of conidia, size of conidia (33.1-196 x24-78 µm) and length of beak (7.1-88 µm) (Fig 3).

Moreover, *A. alternata* species produce mycotoxins and allergens, which may cause problems in the food industry and agriculture as well as in health services (Pavon, 2010). Similarly Ramjegathesh and Ebenezar, (2012) concluded that the *A. alternata* can cause blight in onion and found similar disease symptoms of the sesame. Meena *et al*., (2012) reported the *Alternaria brassicae* morphological features and they were similar in morphological characters of *A. alternata* and other species but not at the conidial shape and septation of conidia. Current and previous reports indicated that *A. alternata* is a primary pathogen causing destructive leaf blight on susceptible crops.

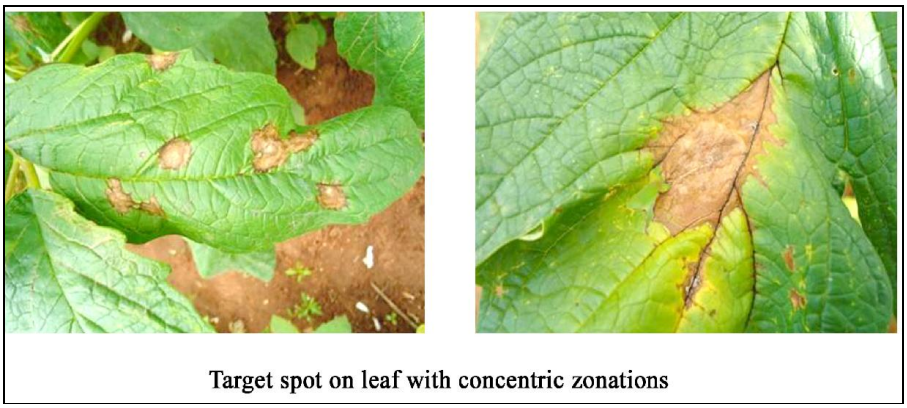

Fig 1. Expression of symptoms of *Alternaria* on leaves of Sesame Cv. E8

Fig. 2. Cultural variability of *Alternaria* species isolated from sesame of different geographical regions of India

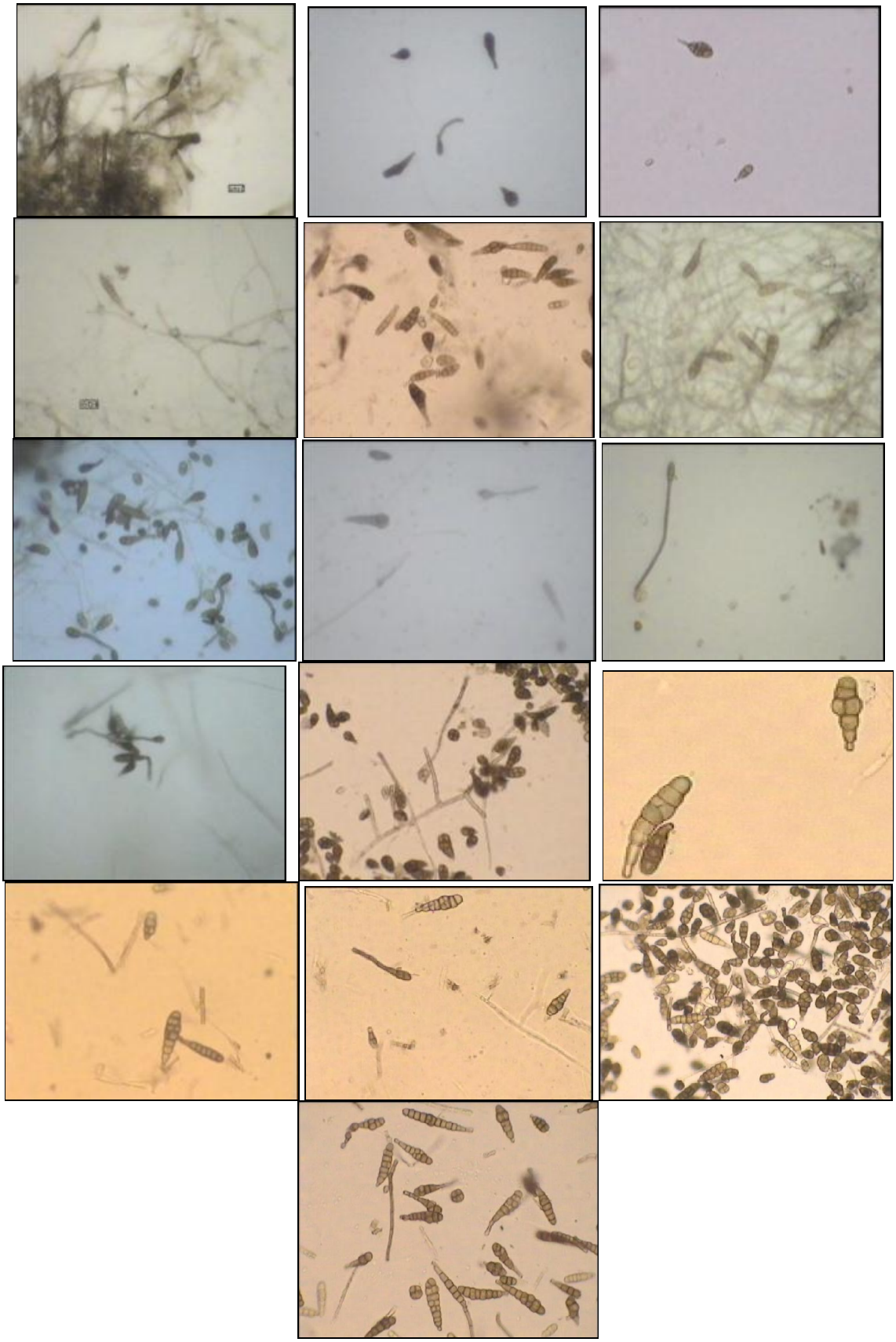

Fig 3. Sporulation pattern of *Alternaria* species isolated sesame of from different geographical regions of India

Pathogenicity tests

The representative isolates from different geographical regions were inoculated by detached leaf technique and maintained under plant growth chamber. The observations were recorded on number of lesions per leaf, the size of the lesion and the time taken for formation of lesions. Out of 164 isolates, twenty one isolates were highly virulent mostly dominated by *A. alternata* (Alt-3, 8, 12, 19, 25, 33, 36, 44, 47, 48, 50, 55, 80, 87, 89, 97, 124, 126, 127 and 137) and nine species were moderately virulent and fourteen were less virulent (Alt- 67, 94, 77, 70, 83, 111, 118, 125, 128, 155, 157, 159, 160 and 161). Similar kind of pathogenic variability of *Alternaria* was recorded by Meena *et al*., (2010) on mustard. In our studies, fungus-free, culture filtrates caused necrotic lesions similar to those produced by the fungus under natural conditions, indicating that a toxin is involved in host colonization and infection establishment.

The role of toxin in pathogencity is supported by other reports on *Alternaria* toxin involvement in all stages of infection, from initial penetration of the tissue to establishment, colonization and death of plant tissue. The same cultivars that were susceptible to the fungus were also sensitive to the toxin, indicating host specificity. Correlation of host resistance and susceptibility with sensitivity to *A. alternata* host specific toxins has been reported in citrus diseases.

DNA extraction and amplification

DNA was extracted from *Alternaria* using CTAB method and purified DNA was amplified using ITS - 1 and ITS – 4 primer set. Out of 164 isolates of *Alternaria* 143 were amplified at 600 bp and the amplified products were checked on 1.5% Agrose gel electrophoresis for further confirmation (Fig 4). All the sequences were confirmed with NCBI BLAST database and revealed that the all the species were matched with reference strains of *Alternaria* species in their homology and identity of the isolates.

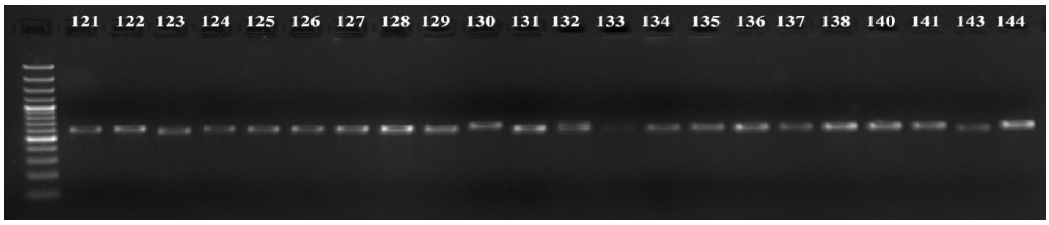

Fig 4. Amplification of ITS r DNA region at 600bp size of *Alternaria* species isolated from Sesame leaves

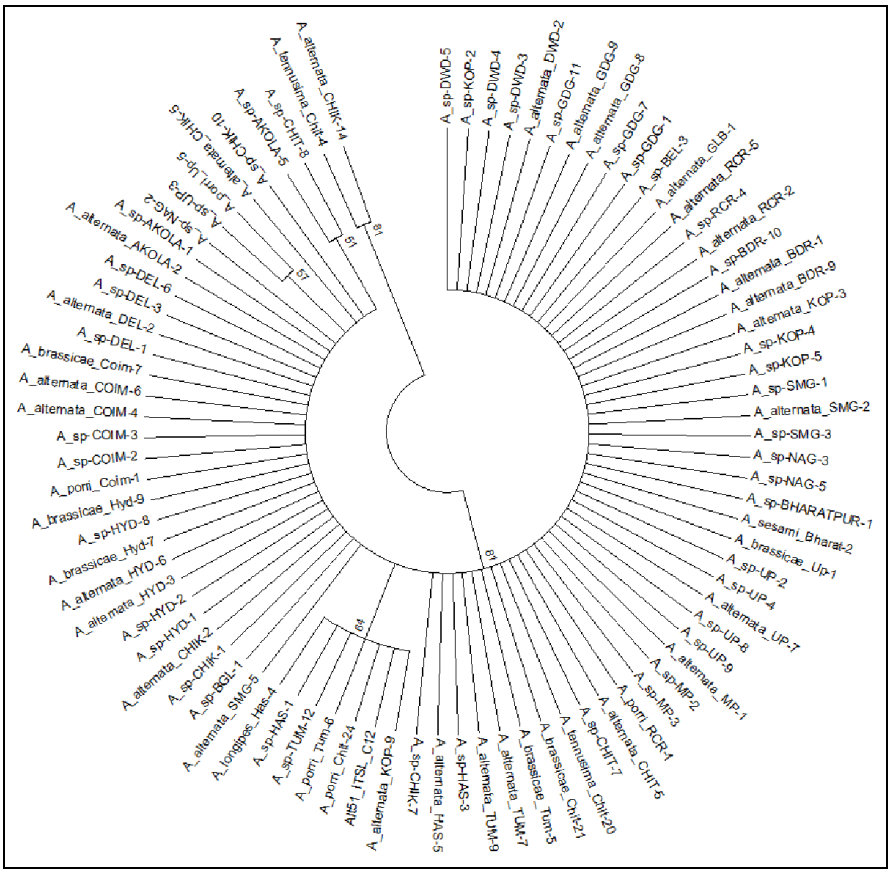

Fig 5. Phylogenetic analyses of *Alternaria* species based on ITS r DNA region

Alternaria species were identified as *Alternaria* spp. (72), *A. alternata* (39), *A. brassicae* (10), *A. porri* (06), *A. tenuissima* (03), *A. sesami* (01), *A. longipes* (12) and the remaining 21 isolates were not amplified. Out of 164 isolates of *Alternaria* 70 per cent fall in one cluster and 30 per cent showing divergence with different nodes (Fig 5). All the *Alternaria* strains sequences were deposited in NCBI genbank along with location of the isolates. Pavon *et al*., (2010) reported the rapid detection of *Alternaria* species from foods based on molecular PCR based approaches and Alt a 1 gene has been developed from DNA of *Alternaria spp. A. alternata, A. porri, A. radicina,* and *A. infectoria.* Similarly Mmbaga *et al*., (2011) reported that the identification *A. alternata* from syringa species causing blight and based on ITS region and synthesis of new species specific primers, *Alternaria* species can be identified. Pryor *et al*., (2002) reported the similar morphological features and it is clearly shown that species of *Alternaria* were ubiquitous and colony characters and pathogencity of fungus may vary based on the crops and media.

RAPD PCR amplification

A total of 19 primers were selected after screening of 25 primers for RAPD analyses. The concentration of the DNA was checked using Nanodrop and based on concentration DNA was standardized for RAPD analyses. All the isolates were clearly amplified with 19 primers and produced different banding pattern of *Alternaria* species of each gene of diversity (Supplementary Fig 1). The diversity of the *Alternaria* species varied from species to species. Among them some produced entirely different bands with species specificity (Alt-90) with OPM-1 at 400bp (Fig 6). Sharma *et al*. (2013) reported the identification of *Alternaria* fungus based on morphological characters is difficult and PCR based detection method was developed and species specific markers were developed and found similar amplification and banding pattern of *Alternaria* species. Roberts *et al*. (2000) reported the small spored *Alternaria* species cannot be differentiated through morphological methods but RAPD method has given accurate and specific differences within diversity of *Alternaria*. Morris *et al*. (2000)

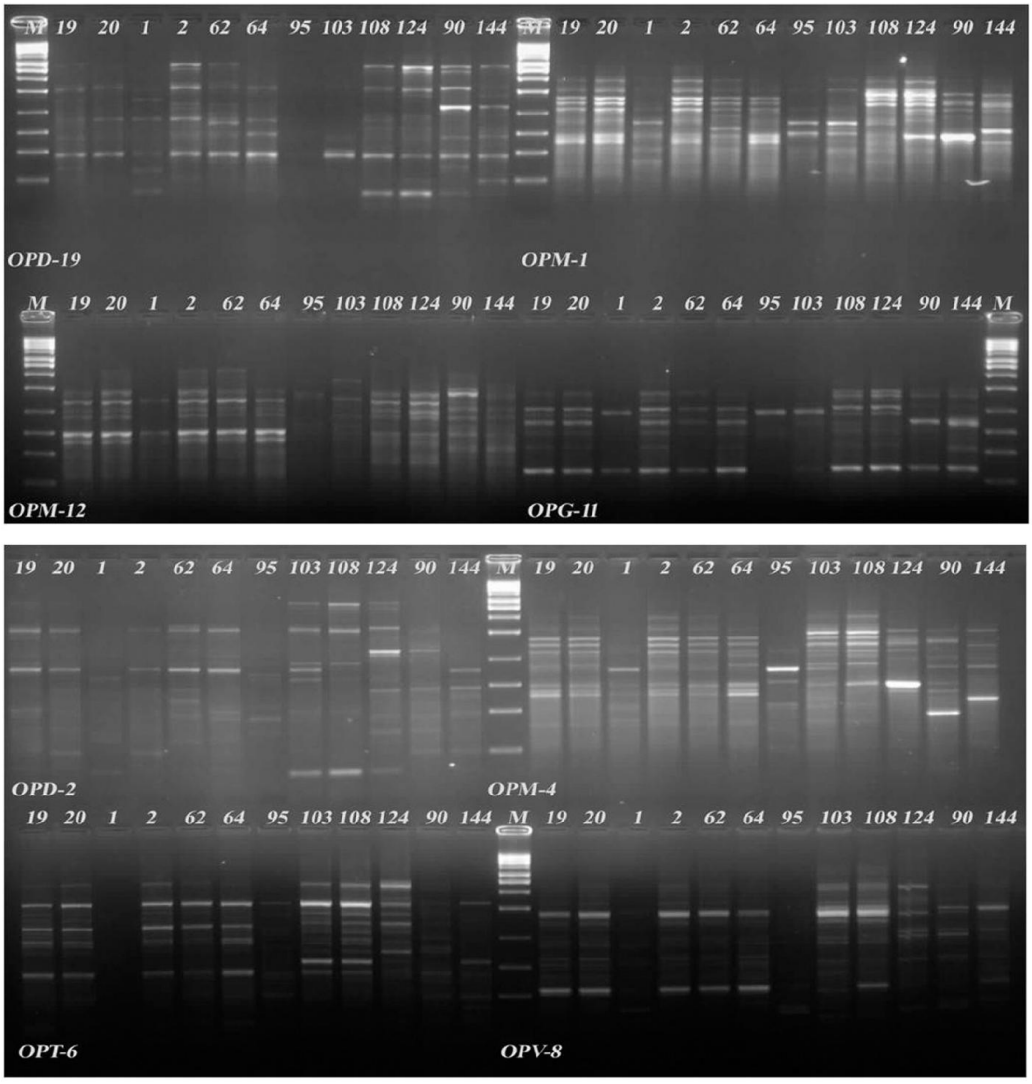

Fig 6. Gel electrophoresis showing Amplification of *Alternaria* species using different RAPD primers

studied the genetic diversity of *A. alternata* isolated from tomato fruits and differentiated the diversity by RAPD analyses.

Among all the species, *A. sesami* produced a species specific band at 400bp with OPM primer but none of the above isolates showed this type of banding pattern. The species specific band of *A. sesami* may be differentiable element among all the bands and sequence pattern of this species band varied from other *Alternaria* species (Supplementary Fig 1).

Cluster analyses

After scoring of bright and clearly visible bands, statistical analysis of the data was performed using the NTSYS-pc program. Clustering of similarity matrices was by UPGMA and the projection of phenograms was done using the TREE program of NTSYS-pc. A Q-type PCA was also performed using NTSYS-pc as an independent test of the clustering of isolates by UPGMA. The diversity of the each isolates was obtained with different polymorphism. The phylogenetic tree showed that a very slight genetic variation was observed among the species (Fig 7 and 7a).

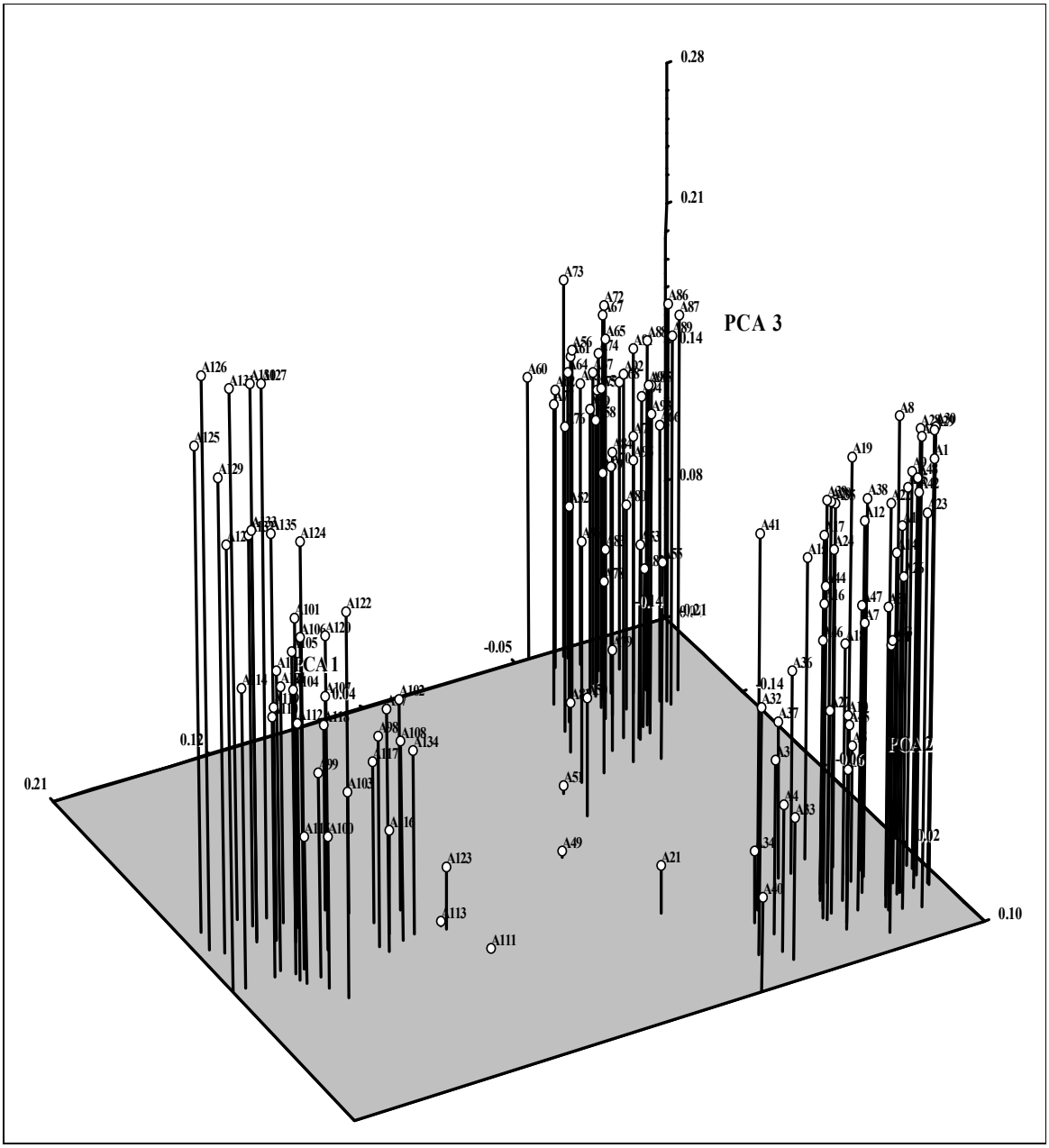

Fig 7. Clustering of similarity matrices of UPGMA and phenograms of *Alternaria* isolates by NTSYS-pc.

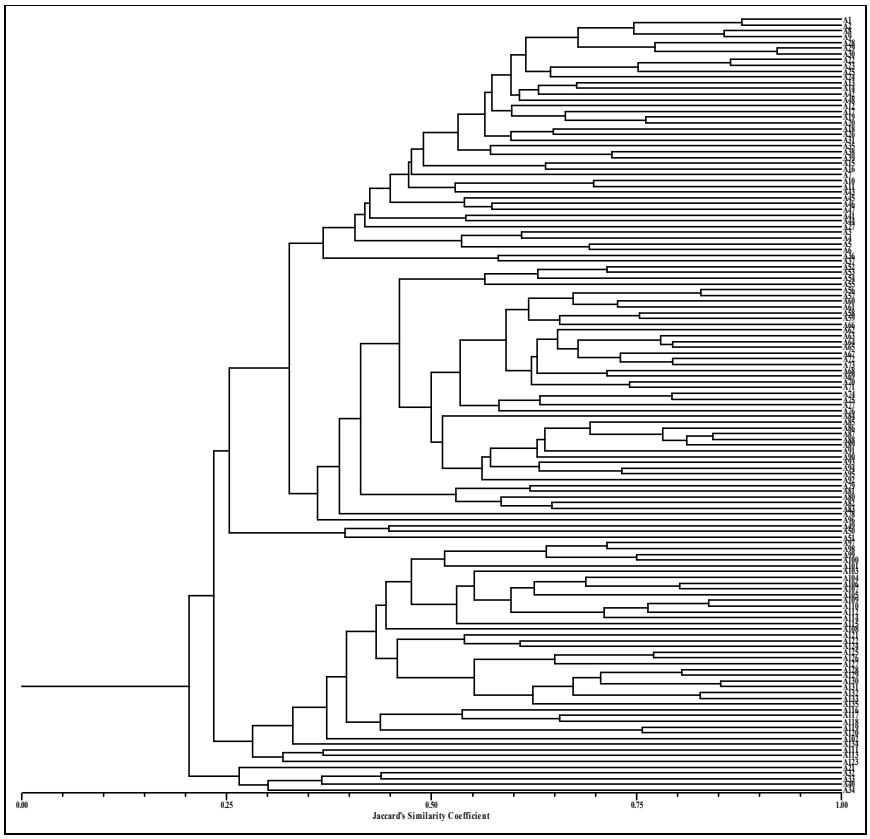

Fig 7a. Phylogenetic analyses of *Alternaria* species based on RAPD method

Table 1. Composition of Alternaria species confirmed based on morphological characters

Isolate	Accession number	Colony color	Topography	Type of margin	Growth rate	Sporulation	Pathogenicity
A. spp.	KF193431	Light grey	Fluffy	Regular	Fast growing	Highly	Highly
A. alternata	KF193432	Light brown	Smooth	Regular	Fast growing	Low	Highly
A. brassicae	KF193454	Light brown	Fluffy	Regular	Moderately	Low	Moderately
A. porri	KF193435	Brown	Fluffy	Regular	Moderately	Low	Moderately
A. tenuissima	KF193437	Grey	Smooth	Regular	Fast growing	Moderate	Highly
A. longipes	KF193441	Grey	Smooth	Regular	Fast growing	Low	Moderately
A. sesami	KF193476	Brown	Smooth	Regular	Fast growing	Low	Moderately

Table 2. A combinations of different *Alternaria sesami* primers were successfully designed and were synthesized and validation of *Alternaria sesami* for identification purposes

Primer Position	Forward Primer	Tm	Primer Position	Reverse Primer	Tm
4	5'-GACCGAGTGGAGTGATTACAGGC	55.9	687	5'-GACCGAGTTCAGCTTGTGTGTCG	56
21	5'-ACAGGCGTCTAGACTCATGTGACG	55.6	651	5'-ATCAAGATGTTCTTCTTCATCTCCC	55.3
62	5'-GAGTGTGTCTGACAGAAGATGTATCCG	56.3	603	5'-CATAAACTCTCCATCCTCTGTGACG	56
86	5'-CCGGGTAGTGATCTTCGTGTGG	57	602	5'-AAACTCTCCATCCTCTGTGACGC	55.6
87	5'-CGGGTAGTGATCTTCGTGTGGG	57	590	5'-TGTGACGCCGAGGATTTCGC	58.6
89	5'-GGTAGTGATCTTCGTGTGGGAGG	55.9	573	5'-TTCGCTCTCTAATTGGCTGTGC	56.4
111	5'-GTTCACTTTTGACCATGTTCTGTCC	55.2	568	5'-TCTCTAATTGGCTGTGCTTGGC	56.2
139	5'-TCTATCGTCACATTCGTCTGTGTGG	55.8	549	5'-TGGCTTTCTTTGATTTCTTCTCC	55.7
144	5'-CGTCACATTCGTCTGTGTGGACG	56.1	512	5'-CCTTCTTGAACTTCTTCTTATGTTTCAGGG	65
155	5'-TCTGTGTGGACGATGGAGAGAGG	52.2			
165	5'-GACCGAGTTCAGCTTGTGTGTCG	57			
176	5'-GGATTCTTCGCCCGTGCAGC	56			

These specific primers allow PCR-based detection and identification of the *A. alternata* pathogen, even without *Alternaria* spores. Similarly Guo *et al*. (2004) reported *A. alternata* genetic variations by using RAMS (random amplified microsatellites) and diversity was analyzed with NTSYS- pc software. The PCR-based diagnostic detection in this study provides a fast and reliable tool to identify and detect *A. alternata* pathogenic isolates that cause lilac leaf

blight. Roberts *et al*. (2000) reported the 264 small spored *Alternaria* species and cluster analyses showed the variability among the species. Similarly Guo *et al*. (2004) reported cluster analyses *A. alternata* variation can assess through Dendrogram and found that even 1% variation was also computed.

SCAR marker development

A total of 25 primers were used for screening and out of 25 primers OPA, OPD, OPM, OPG, OPT and OPV primers were selected for SCAR marked detection for the species specific band length of the species is around 750 bps (Fig 6). The cloned and selected colonies were sent for sequencing of the desired species specific gene and after getting the sequence results, we will be able to synthesize the species specific marker or primer for *A. sesami* causing blight in sesame.

Sequencing of Positive Clones

After cloning of insert, the three positive cloned colonies were sequenced and the based on the insert and the sequence data primers were synthesized for validation of the insert or the required specific primer. *A. sesami* species specific genes have been cloned and were sequenced for SCAR marker development. Around 300 primer combinations were obtained but out of those only 25 combinations were selected (Supplementary Fig 2). 25 sets of primers were successfully designed and were synthesized and validation of these primers with *A. sesami* under process (Table 2).

Conclusion

A. sesami causing blight on sesame was identified based on morphological and molecular studies. The species specific primers have been identified and different set of primers have synthesized by using these newly synthesized primers *A. sesami* can be directly identified by single PCR reaction.

Acknowledgement

The authors are thankful to ICAR, New Delhi, Govt. of India for funding of Out Reach Project on Leaf spots diseases of Horticulture crops and Dr. K. V Bhat, NRC on DNA Finger Printing, NBPGR, New Delhi for providing valuable suggestions and laboratory facilities for the development of SCAR marker for *A. sesami* during the research work.

REFERENCES

Amaresh YS and Nargund VB. 2005. Isolation of toxin from *Alternaria helianthi* causing blight in sunflower. *Journal Mycopathology and Plant Pathology*. 35: 367-369

Berry SZ. 1960. Comparison of cultural variants of *Alternaria sesami*. *Phytopathology*. 50: 298-304.

Bhaskaran R and Kandaswamy TK. 1978. Production of a toxic metabolite by *Alternaria helianthi in vitro* and *in vivo*. *The Madras Agriculture Journal*. 65; 1978: 801-804.

Chennappa G, Adkar-Purushothama CR, Suraj U, Tamilvendan K and Sreenivasa MY. 2013. Pesticide tolerant *Azotobacter* isolates from paddy growing areas of northern Karnataka, India. *World Journal Microbiology and Biotechnology*. 30: 1-7

Guleria S and Kumar A. 2006. *Azadirachta indica* leaf extract induces resistance in sesame against *Alternaria* leaf spot disease. *Journal Cell and Molecular Biology*. 5: 81-86

Guo LD, Xu L, Zheng WH and Hyde KD. 2004. Genetic variation of *Alternaria alternata* an endophytic fungus isolated from *Pinus tabulaeformis* as determined by random amplified microsatellites (RAMS). *Fungal Diversity*. 16:53-65

Khodadadi F, Panjehkeh N, Ahmadinejad and Aminaee MM. 2011. Genetic variability of toxigenic and non toxigenic isolates of *Aspergillus flavus* and *Aspergillus parasiticus* by RAPD marker. *Journal of Food Safety*. 31: 439-444

Lubiana AS and Murugan K. 2012. Biochemical characterization of oxidative burst during interaction between sesame (*Sesamum indicum* L.) in response to *Alternaria sesami*. *International Journal of Advanced Biological Research*. 2: 86-91

McKay GJ, Brown AE, Bjourson AJ and Mercer PC. 1999. Molecular characterization of *Alternaria linicola* and its detection in linseed. *European Journal of Plant Pathology*. 105: 157-166

Meena PD, Awasthi RP, Chattopadhyay C, Kolte SJ and Kumar A. 2010. *Alternaria* blight: a chronic in Rapseed mustard. *Journal of Oilseed Brassica* 1: 1-11

Meena PD, Rani A, Meena R, Sharma P, Gupta R and Chowdappa P. 2012. Aggressiveness, diversity and distribution of *Alternaria brassicae* isolates infecting oilseed *Brassica* in India. *African Journal of Microbiological Research*. 6: 5249-5258

Mmbaga MT, Shi A and Kim MS. 2011. Identification of *Alternaria alternata* as a causal agent for leaf blight in Syringa species. *Plant Pathology Journal*. 27:120-127

Morris PF, Connolly MS and Clari DS. 2000. Genetic diversity of *Alternaria alternata* isolated from tomato in California assessed using RAPDs. *Mycological Research*. 104: 286-292

Ostry V. 2008. *Alternaria* mycotoxins: An overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin Journal*. 1(2):175-188.

Pavon MA, Gonzalez I, Pegels N, Martin R and Garcia T. 2010. PCR detection and identification of *Alternaria* species-groups in processed foods based on the genetic marker Alt a-1. *Food Control* 21:1745-1756

Pryor BM and Michailides TJ. 2002. Morphological, pathogenic, and molecular characterization of *Alternaria* isolates associated with *Alternaria* late blight of pistachio. *Ecological and Population Biology*. 92:406-416

Rajpurohit TS. 2011. Diseases of Niger and their management. *Plant Science Feed*. 1: 19- 22

Ramjagathesh R and Ebenezar EG. 2012. Morphological and physiological characters of *Alternaria alternata* causing leaf blight diseases in onion. *International Journal of Plant Pathology*. 3: 34-44

Rao RN and Vijayalakshmi M. 2000. Studies on *Alternaria* sesami pathogenic to sesame. *Microbiological Research*. 155:129-131.

Roberts RG, Reymond ST and Andersen B. 2000. RAPD fragments pattern analyses and morphological segregation

of small spored *Alternaria* species and groups. *Mycological Research*. 104:151-160

Savitha AS. Naik MK and Ajithkumar K. 2012. *Alternaria sesami* causing blight of sesame produces toxin and induces the host for systemic resistance. *Asian Journal of Research in Chemistry*. 5:176-1181

Sharma P, Deep S, Sharma M, Bhatti DS and Chowdappa P. 2013. PCR based assay for the detection of *Alternaria brassicicola* in crucifers. *Indian Phytopathology*, 66 (3): 263-268

Shindhe GG, Lokesha R, Naik MK and Ranganath AGR. 2011. Inheritance study on phyllody resistance in sesame (*Sesamum Indicum* L.). *Plant Archives*. 11: 775-776

Tiwari S, Kumar S and Gontia I. 2011. Biotechnological approaches for sesame (*Sesamum indicum* L.) and Niger (*Guiotia abyssinica* L.f. Cass). *Asian Pacific Journal of Molecular Biology and Biotechnology*. 19:2-9

White TJ, Bruns T, Lee S and Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocols: A guide methods and amplification. Academic press, San Deigo, USA, PP 315-322.

Woody MA and Chu FS. 1992. Toxicology of *Alternaria* mycotoxins. In: Chełkowski, J. and Visconti, A. (Eds.) Topics in secondary metabolism, vol. 3, *Alternaria*: biology, plant diseases and metabolites. Elsevier, New York, USA, 409-434.

Yu SH, Mathur SB and Neergaard P. 1982. Taxonomy and pathogenicity of four seed borne species of *Alternaria* from sesame. *Transaction British Mycology Society*. 78:447-458.

Supplementary Sequence Data

Compiled data of the sample D19.CLONE 1 with T7U20 & SP6 primer

>A05_33B.UAS.MNN.D19.1.T7U20_2014-02-07_1.ab1

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>E05_37B.UAS.MNN.D19.1.SP6_2014-02-07_1.ab1

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Reverse complement of :E05_37B.UAS.MNN.D19.1.SP6_2014-02-07_1.ab1

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Compiled sequence data

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Insert Sequence

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Alignment of pGEM with A05_33B.UAS.MNN.D19.1.T7U20_ 2014-02-07_1.ab1

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Compiled data of M13U18 & M13R20

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Alignment of pGEM with A07_49A.UAS.MNN.D19.1.M13U18_ 2014-02-07_1.ab1

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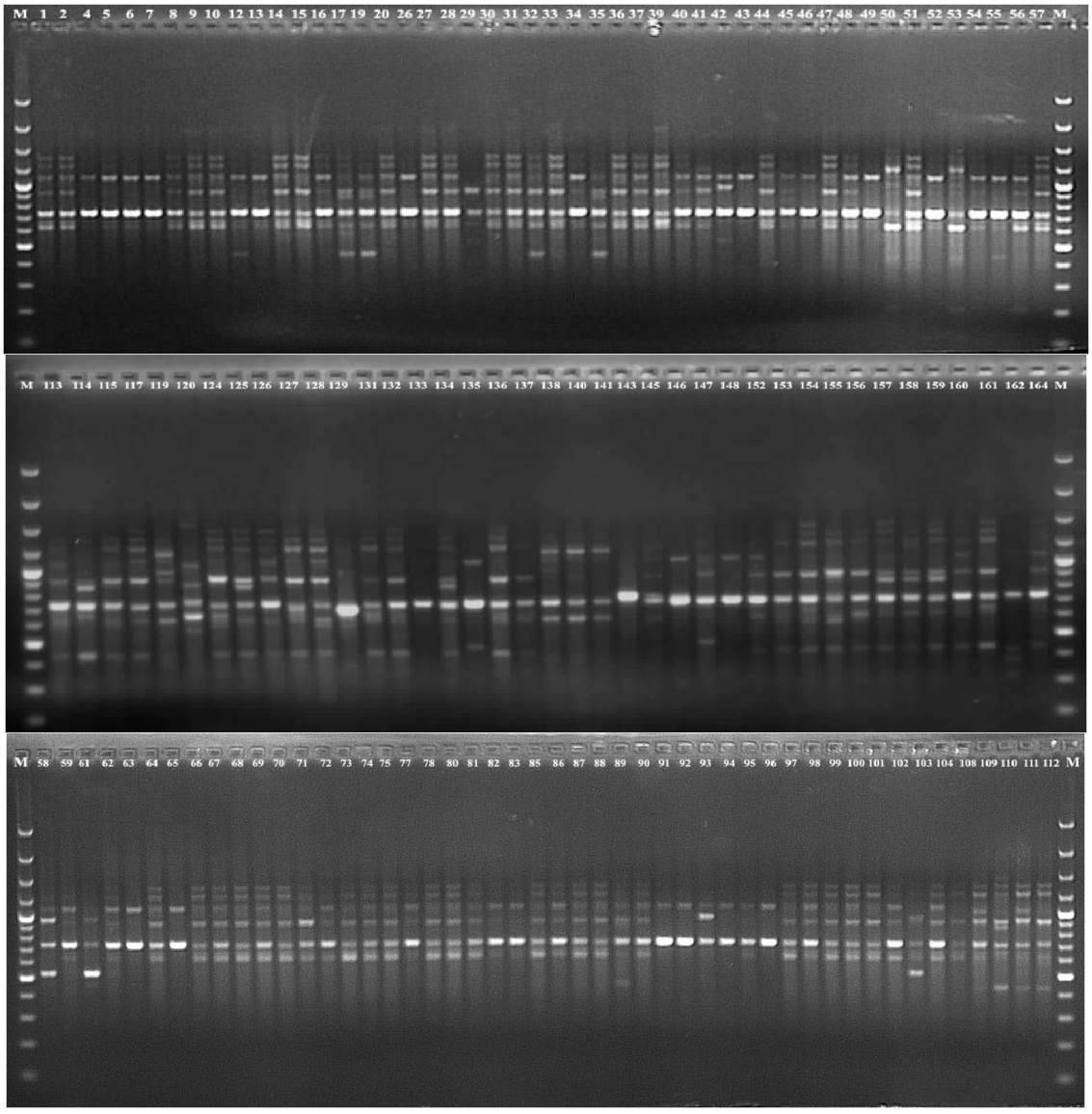
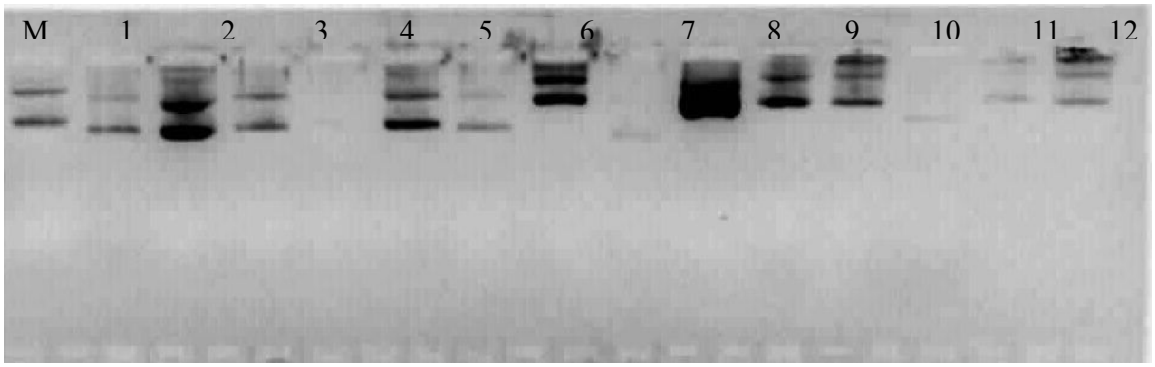
Supplementary Figures



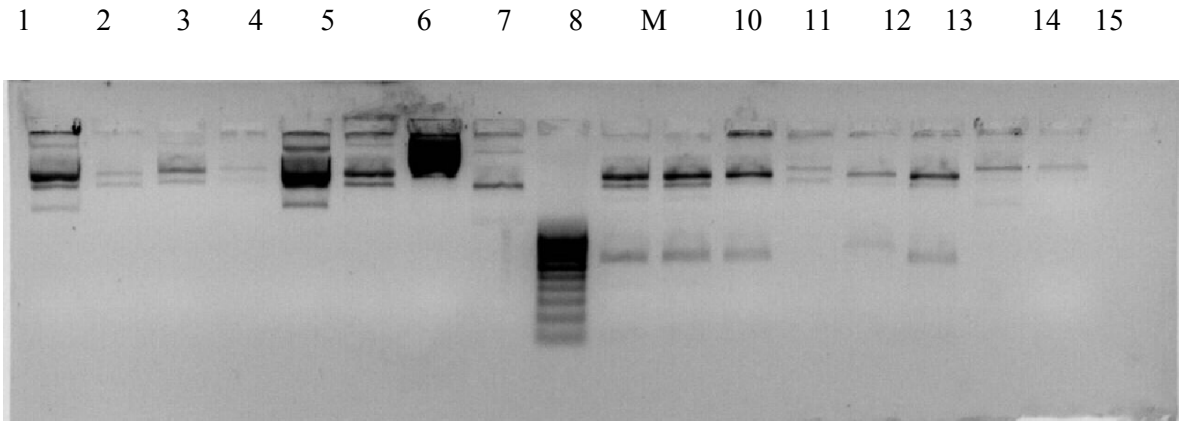
Supplementary Fig 1.



Lane Description

Lane M : Control DNA puc18
Lanes 1-8 : Clones of sample A13(1-8 nos)
Lanes 9-14 : Clones of sample D19 (1-6 nos)

Confirmation of clone by Restriction digestion using ECOR1



Lane Description

Lane M : 100bp Step up DNA Ladder
Lanes 1-8 : RE digested Clones of sample A13 (1-8 nos)
Lanes 10-17 : RE digested Clones of sample D19 (1-8 nos positive clones)

Supplementary Fig 2.

****