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Molecular Characterization of *Aspergillus niger* Van Tieghem Isolates by Using RAPD Markers

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ABSTRACT

Background: *Aspergillus niger* Van Tiegham is a filamentous, ubiquitous and cosmopolitan fungus, commonly known as “black mould” or black aspergilli. *A. niger* is responsible for causing diseases in plants, humans and animals. *A. niger* causes black mould disease during post-harvest stage of garlic bulbs. The potential of RAPD technique for identifying DNA marker related to the intraspecific diversification of the pathogen led us to instigate the genetic diversity within *A. niger.*

Methods: A PCR-based technique, involving the random amplification of polymorphic DNA (RAPD), was used for assessing genomic variability among the ten isolates of *A. niger* isolated from infected garlic bulbs. RAPD- PCR conditions were optimized for eight primers of series OPA and POC out of 40 total primers.

Result: RAPD results were evaluated by a statistical software Minitab and a phylogenetic tree was prepared. The primer OPA-8, OPA-12, OPC-1, OPC-2, OPA-7 and OPC-18 showed 100 per cent polymorphism, among the *A. niger* isolates.

Key words: *Aspergillus niger*, Characterization, Garlic, RAPD.

INTRODUCTION

Garlic (*Allium sativum* L.) is the most important bulbous crop after onion, belonging to *Amaryllidaceae* family (Sharma, 2012). Black mould rot of garlic is caused by *A. niger*, which is a filamentous, ubiquitous and cosmopolitan fungus, commonly known as “black mould” or black aspergilli. This fungus is among the most common fungi causing food spoilage and biodeterioration of other materials (Samson *et al.,* 2004). Strains of *A. niger* are used in the manufacture of several acids like citric, gluconic and itaconic acids and enzymes, like glucose oxidase and lysozyme. Over 99% of total global production of citric acid is through fermentation by *A. niger* (Dube, 2013). Being saprophytic and filamentous in morphology, *A. niger* resides and perpetuates in soil, forage, organic debris and food products. The most favourable temperature conditions for the growth of the fungus are 28°C-34°C followed by the worm and moist conditions eliciting infection (Tysony and Fullerton, 2004). However, *A. niger* has been found to be an opportunistic because of various infections of human beings. It can be dangerous to humans if intense spores inhaled, by causing severe lung problems *i.e.* Aspergillosis. This fungus can also cause ear infections, which ultimately damage ear canal and tympanic membrane (Schuster *et al.,* 2002). *A. niger* causes black mould disease during post-harvest stage of garlic bulbs (McDonald *et al.,* 2004). The modified polymerase chain reaction (PCR) with single primers of arbitrary nucleotide sequence and requiring no prior sequence information have proved useful in detecting intraspecific polymorphism among organisms (Welsh and Mc Clelland, 1990 and Williams *et al.,* 1990). This amplification technique (arbitrarily primed PCR on RAPD) can generate specific DNA fragments useful for genome mapping, identification of isolates and application in molecular biology (Hadrys *et al.,* 1992). The potential of this technique for identifying DNA marker related to the intraspecific diversification of the pathogen led us to instigate the genetic diversity within *A. niger.*

In the present study we have characterized ten different isolates of *A. niger.* The aim of the present study was to characterize isolates of the fungus at molecular level that leads to further elucidation of genetic diversity.

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MATERIALS AND METHODS

Source of A. niger isolates

Ten isolates of *A. niger* which were isolated from infected garlic bulbs, collected from different regions of India listed below in Table 1.

Genomic DNA extraction from fungal mycelium

DNAs of 10 isolates of *A. niger* were extracting using a CTAB method as described by Leisova *et al.,* (2005) with some modifications.

Random amplification of polymorphic DNA (RAPD) analysis

For the molecular characterization of A. *niger* isolates, the eight different 10-mer RAPD primers were screened for the amplification of template DNA of *A. niger* (Table 2). The primers that gave clear and polymorphic amplification patterns were used for further analysis of all the 10 isolates of *Aspergillus niger.* Amplified DNA fragments for each accession were scored as present (1) or absent (0). Data generated by eight RAPD primers were used to compile a binary matrix for cluster analysis. Genetics similarity among accessions was calculated according to Jaccard's coefficient (Jaccard, 1908). The present study was carried out at B.A. College of agriculture, department of plant pathology, anand agricultural university, anand during 2015-16.

Table 1: Source of *A. niger* isolates.

Name of Isolate	Place of collection	District/State
AN-1	Talaja	Bhavnagar
AN-2	Sardar Patel, Market	Anand
AN-3	Vadodara	Vadodara
AN-4	Surat	Surat
AN-5	Vasi (Mumbai)	Maharastra
AN-6	Barvav, Idar	Sabarkantha
AN-7	Dwarka	Dwarka
AN-8	Amreli	Amreli
AN-9	South West Delhi	New Delhi
AN-10	Kasargod	Kerala

Table 2: List of primers used for the RAPD analysis.

Primer	Sequence (5'-3')	Bases
OPA 8	GTGACGTAGG	10
OPA 12	TCGGCGATAG	10
OPA 18	AGGTGACCGT	10
OPC 1	TTCGAGCCAG	10
OPC 2	GTGAGGCGTC	10
OPC 7	GTCCCGACGA	10
OPC 8	TGGACCGGTG	10
OPC 18	TGAGTGGGTG	10
Total		80

RESULTS AND DISCUSSION

Random Amplification of Polymorphic DNA (RAPD) study

In recent years, molecular markers have received arable attention and have been used for genetic diversity phylogenetic, evolutionary, mapping and tagging of agronomically important traits in different plant species. It can also be used in the study of molecular diversity in microorganism such as fungus, bacteria, viruses etc. In this study, the present investigation was carried out with the ten different isolates of *A. niger* to study the molecular variability in these isolates. Therefore, this technique was employed to detect variability present among ten isolates *i.e.* AN-1, AN-2, AN-3, AN-4, AN-5, AN-6, AN-7, AN-8, AN-9 and AN-10 of *A. niger* (Plate 1a, 1b and 1c).

The present study showed a high level of genetic variability between the isolates of *A. niger*. Total 40 different 10-mer RAPD primers of OPA and OPC series (Eurofins Genomics India Pvt. Ltd.) were screened for the amplification of template DNA of *A. niger.* Among these, 32 primers did not produced amplification at all, while only eight primers were able to produce clear banding patterns and were subsequently used to analyze the entire set of 10 isolates of *A. niger*. The fragment size was detected by comparing the amplicons with a 100 bp Ladder. The highest polymorphism (100%) was recorded by the primer OPA-8, OPA-12, OPC-1, OPC-2, OPA-7 and OPC-18. Whereas, the lowest polymorphism was observed in the primer OPC-8 (97.29%) (Table 3).

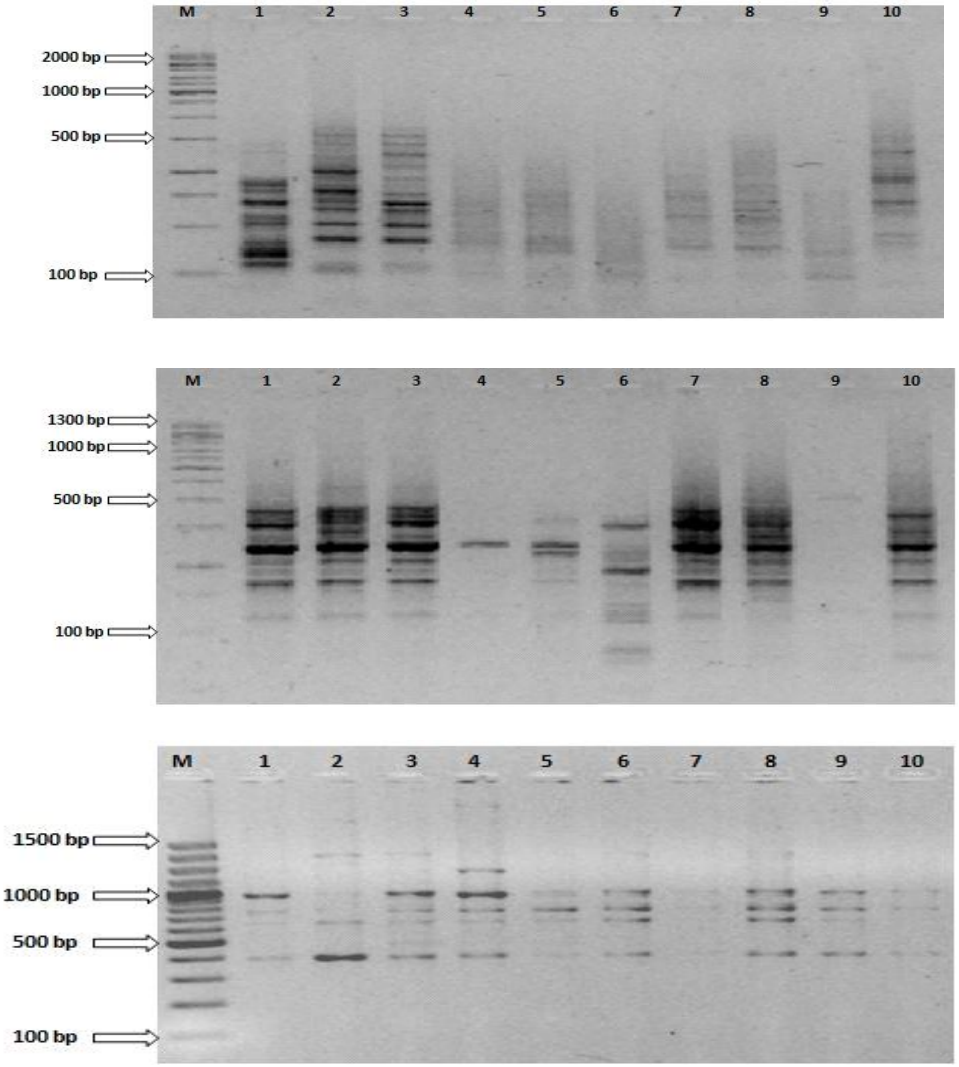
Analysis of the DNA of ten isolates with eight primers showed amplification of a total 360 bands, with a range of 34 to 59 bands per primer. Overall, *A. niger* isolates exhibited

Table 3: Details of amplification obtained with different RAPD primers.

Name of primer	Annealing temperature	No of loci	No. of poly morphic loci	Polymorphism (%)	Total no. of bands	PIC *value
OPA 8	38	20	20	100	59	0.939
OPA 12	36	13	13	100	54	0.887
OPA 18	36	10	09	97.50	40	0.831
OPC 1	36	11	11	100	34	0.860
OPC 2	36	14	14	100	43	0.910
OPC 7	36	08	08	100	43	0.837
OPC 8	36	07	06	97.29	37	0.818
OPC 18	36	14	14	100	50	0.893
Total		97	95	-	360	6.975
Average		12.13	11.87	99.35	45	0.872

a moderate level of genetic diversity. The maximum 59 bands were generated by a single primer (OPA-8); whereas, OPA-12, OPA-18, OPC-1, OPC-2, OPC-7, OPC-8 and OPC-18 generated 54, 40, 34, 43, 43, 37 and 50 bands, respectively. The lowest number of bands was generated by primer OPC-1 (34 bands). These results showed potentiality of RAPD to discriminate among isolates and suggested their application for species identification. The purpose of this study was to identify the specific primers which are likely to be efficient in revealing the diversity among the isolates of *A. niger.*

Dendrogram (Fig 1) based on "Jaccard (1908) unbiased measures of genetic distance (Table 4) by UPGMA method" formed two clusters namely A and B. Cluster A was divided into two sub-clusters A1 and A2. Sub-cluster A1 was further divided into A11 and A12 clusters, respectively. Cluster A11 having five isolates AN-1, AN-7, AN-8, AN-2 and AN-3 collected from Bhavnagar, Dwarka, Amreli, Anand and Vadodara, respectively. Cluster A12 consisted AN-10 isolate collected form Kerala, while, cluster A2 consisted AN-6 and AN-9 isolates which were collected from Sabarkantha and New Delhi. Cluster B includes AN-4 and AN-5 isolates which were collected from Surat and Maharastra. The similarity coefficient ranged from 0.18 to 0.55 with all the eight primers. Highest similarity (0.55) was observed between AN-2 and AN-3 isolates, which were collected from Anand and Vadodara respectively, while lowest similarity (0.18) was observed between AN-2



[M-100+bp; 1.AN-1 (Bhavnagar), 2.AN-2 (Dwarka), 3.AN-3 (Amreli), 4.AN-4 (Anand), 5.AN-5 (Vadodara), 6.AN-6 (Kerala), 7.AN-7 (Sabarkantha), 8.AN-8 (New Delhi), 9.AN-9 (Surat), 10.AN-10 (Maharastra)]

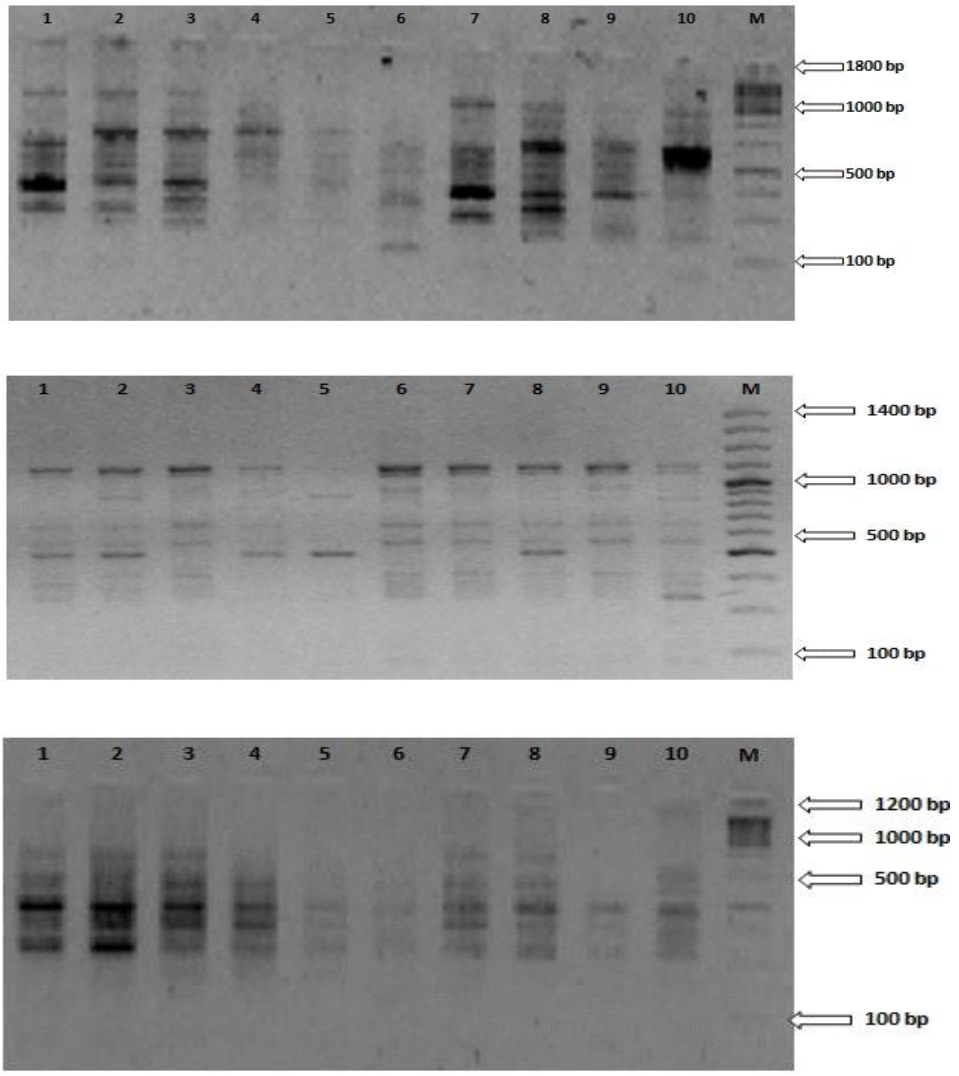
Plate 1 (a): RAPD amplification patterns of ten isolates of *A. niger* using primers OPA 8, OPA 12 and OPA 18.

and AN-9 isolates which were collected from New Delhi and Anand, respectively.

Results similar to the present findings have been reported by Prajapati *et al.* (2016). He carried out RAPD analysis among the ten isolates of *A. niger* with six primers *i.e.* OPA-5, OPA-8, OPA-12, OPC-2, OPC-7 and OPC-18 showing amplification of 159 bands with a range of 10 to 38 bands per primer. The similarity coefficient ranged from 0.037 to 0.56.

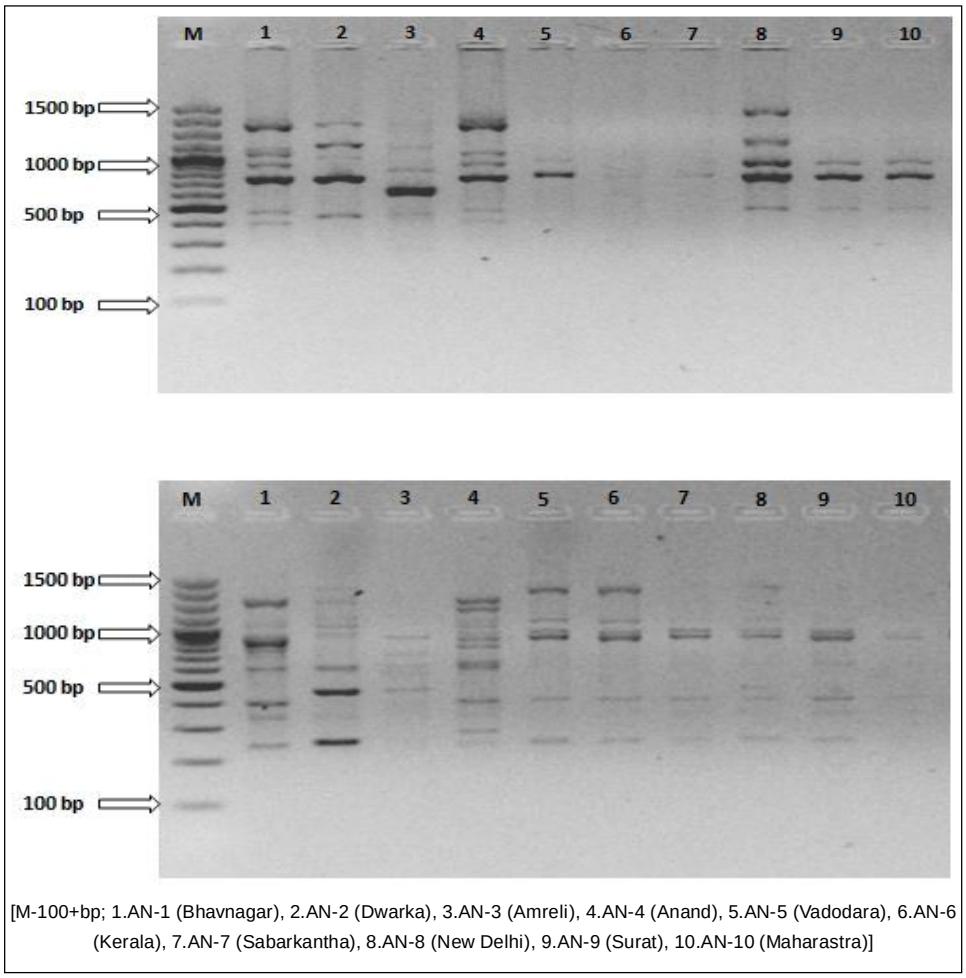
Tawfik *et al.* (2022) reported 78.56 per cent of polymorphism through RAPD-PCR in Mutant *Aspergillus niger* infection in different plants. Rani *et al.,* (2018) studied genetic relatedness among eight virulent isolates of *A. niger* from groundnut by using RAPD analysis and reported that the similarity index values among the *A. niger* isolates varied from 0.571 to 0.229. Abeer *et al.* (2015) they studied molecular characterization of twelve isolates of two species *A. parasiticus* and *A. terreus* by using DNA markers. They found that the RAPD and ISSR analysis revealed a high level of genetic diversity in *A. parasiticus* and *A. terreus* population and RAPD fingerprints of *A. niger*, *A. flavus* and *A. parasiticus* revealed polymorphism in 37, 59, 51 per cent of the analyzed *Aspergillus* sp.

The genomic DNA of ten isolates *A. niger* purified and were subjected to RAPD analysis by using 20 different primers. Out of 20 random primers, primer P2 (5'-ACGGCGTATG-3') was optimized to screen all ten isolates of *A. niger* and showed amplifications of different sizes.



[M-100+bp; 1.AN-1 (Bhavnagar), 2.AN-2 (Dwarka), 3.AN-3 (Amreli), 4.AN-4 (Anand), 5.AN-5 (Vadodara), 6.AN-6 (Kerala), 7.AN-7 (Sabarkantha), 8.AN-8 (New Delhi), 9.AN-9 (Surat), 10.AN-10 (Maharastra)]

Plate 1 (b): RAPD amplification patterns of ten isolates of *A. niger* using primers OPC 2, OPC 7 and OPC 8.



[M-100+bp; 1.AN-1 (Bhavnagar), 2.AN-2 (Dwarka), 3.AN-3 (Amreli), 4.AN-4 (Anand), 5.AN-5 (Vadodara), 6.AN-6 (Kerala), 7.AN-7 (Sabarkantha), 8.AN-8 (New Delhi), 9.AN-9 (Surat), 10.AN-10 (Maharastra)]

Plate 1 (c): RAPD amplification patterns of ten isolates of A. niger using primers OPC1 and OPC 18.

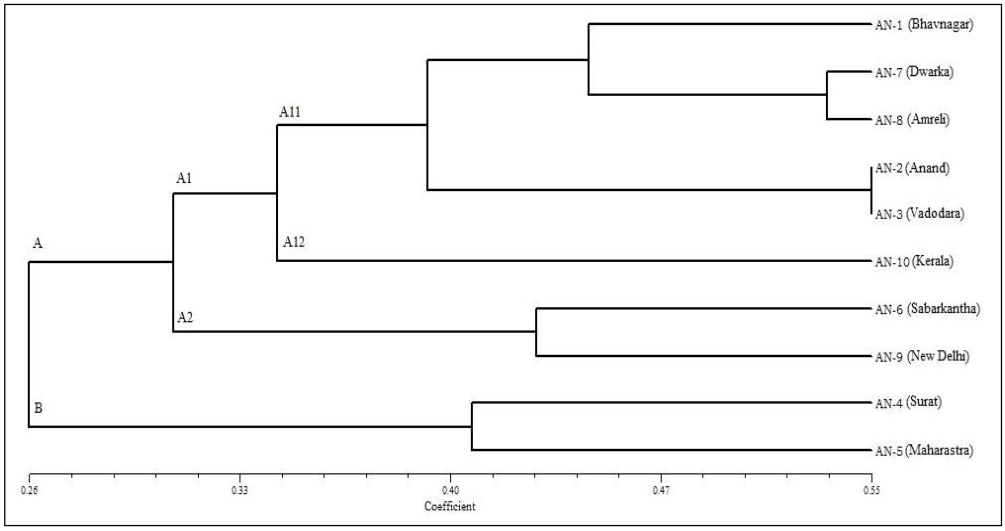


Fig 1: Dendrogram for isolates of *A. niger* based on Jaccard's (1908) similarity coefficient using UPGMA as the clustering method for RAPD.

Table 4: Jaccard's similarity coefficient between isolates of *A. niger* based on RAPD Makers.

	AN-1	AN-2	AN-3	AN-4	AN-5	AN-6	AN-7	AN-8	AN-9	AN-10
AN-1	1.00									
AN-2	0.47	1.00								
AN-3	0.32	0.55	1.00							
AN-4	0.30	0.34	0.24	1.00						
AN-5	0.21	0.26	0.24	0.41	1.00					
AN-6	0.27	0.26	0.25	0.24	0.27	1.00				
AN-7	0.43	0.31	0.36	0.22	0.26	0.37	1.00			
AN-8	0.47	0.45	0.45	0.30	0.30	0.34	0.53	1.00		
AN-9	0.32	0.18	0.19	0.27	0.24	0.43	0.42	0.40	1.00	
AN-10	0.33	0.31	0.36	0.24	0.21	0.28	0.33	0.40	0.40	1.00

Based on amplification patterns, the ten fungal isolates were divided into five groups in dendrogram analysis. Group I contained isolates 658 and 880 (100%), group II have isolates 0074, 840, 1005 and 1109 (68.38%), group III has 506 (55.28%) and IV contained isolates 0002 and 744 (36.76%) and group V included 764 (22.54%) based on genomic similarity percentages (Ishfaq *et al.,* 2014). Irshad and Nawab (2012) studied molecular characterization of seven different species of *A. niger*. RAPD results were evaluated by a statistical software Minitab and a phylogenetic tree was prepared. GL Decamer B-09 showed 38 bands and GL Decamer B-10 gave 46 bands, showing 50 and 57 per cent similarity respectively, among species. Yuan *et al.* (1995) studied similarity between two *Aspergillus* species by using Single primers with arbitrary sequences to generate random amplified polymorphic DNA (RAPD) markers from strains of *A. parasiticus* and *A. sojae.* Three decamers, OPA-04, OPB-10 and OPR-01, allowed adequate discrimination between strains of *A. parasiticus* and *A. sojae* in RAPD analyses. *A. sojae* was further separated into group I and group II with the three primers. On the other hand, *A. parasiticus* was divided into group A and group B when amplified with OPA-04 and OPR-10 primers. The previously misidentified strain CCRC 32423 and the misclassified strain CCRC 30227 were identified as *A. flavus* and *A. sojae*, respectively, on the basis of RAPD patterns and morphological characteristics. Lavkor (2019) reported molecular analysis of fungal isolates from soil, air and infected peanut plants identified *A. flavus* using the β-tubulin gene and PCR-RFLP. Out of 325 isolates, 254 were found to contain aflatoxin biosynthesis genes, with 213 producing aflatoxin. The findings highlight *A. flavus* as the primary species responsible for aflatoxin contamination and suggest that understanding the aflatoxin gene cluster can aid in assessing toxicological risks and selecting biocontrol measures. Tejpal *et al.,* (2022) identified significant cultural and morphological variability among fungal isolates, with isolate ANJP-04 showing the highest virulence. Among ten groundnut varieties tested, RG-644, M-13 and RG-510 were resistant, while others ranged from moderately resistant to highly susceptible, suggesting these resistant varieties should be cultivated to manage collar rot effectively.

Potential application of the findings

The molecular characterization of Aspergillus niger isolates using RAPD markers offers precise identification and differentiation of strains causing black mold disease in garlic. This knowledge enables targeted disease management strategies, including tailored fungicide applications and crop rotation practices. By understanding genetic diversity, farmers can implement more effective measures to control disease outbreaks and enhance crop health. This research also lays the groundwork for developing resistant garlic varieties through selective breeding, promoting sustainable agricultural practices in the face of fungal pathogens. Singh *et al.,* (2017) standardized a real-time PCR method using TaqMan probes to amplify the aflatoxin/sterigmatocystin biosynthesis gene omt-1 in Aspergillus spp. Isolates from 53 aflatoxin-positive feed samples were successfully identified, demonstrating the technique's efficiency in distinguishing toxigenic strains from other molds.

CONCLUSION

The RAPD analysis of ten *Aspergillus niger* isolates revealed significant genetic diversity among the strains responsible for black mould disease in garlic. The study identified eight effective primers that generated a range of polymorphic DNA fragments, enabling the differentiation and classification of isolates into distinct clusters. This genetic variability underscores the potential for targeted disease management strategies and informs the development of resistant garlic varieties. The findings not only enhance our understanding of *A. niger* genetic diversity but also support more effective control measures and sustainable agricultural practices to combat fungal pathogens.

Conflict of interest

The authors declare that they have no conflict of interest regarding the publication of this research. There are no

financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of this study.

REFERENCES

Abeer, R.M., ABD-EL-Aziza, Monira, R., AL-Othmana, Mohamed, A., Mahmoud, Samah, A., (2015). Molecular characterization of *Aspergillus parasiticus* and *A. terreus* producing and non-producing silver nanoparticles using DNA markers. Digest J. Nanomaterials and Biostructures. 10: 31-41.

Bajaya, T., Ghasolia, R.P., Bajya, M., Shivran, M. (2022). Variability and virulence analysis of *aspergillus niger* Isolates causing collar rot of groundnut. Legume Research. 45(7): 914-920. doi: 10.18805/LR-4854.

Dube, H.C. (2013). An introduction to fungi (4th edition), Scientific Publishers (India): 200.

Hadrys, H., Balack, M. and Schrerwater, B., (1992). Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol. 1: 55- 63.

Irshad, S. and Nawab, R., (2012). Molecular characterization of seven different species of *aspergillus niger* through random amplified polymorphic DNA (RAPD) and enzyme analysis. Journal of Microbiology Research. 2: 47-50.

Ishfaq, M., Mahmood, N., Nasir, I.A. and Saleem, M., (2014). Molecular and biochemical screening of local *Aspergillus niger* strains efficient in catalase and laccase enzyme production. Int. J. Agril. Biol.16: 177-182.

Jaccard, P., (1908). Nouvelles researchers sur la distribution florale. Bulletins of Soc. Vaud. Sci. Nat. 44: 223-270.

Lavkor, I. (2019). Molecular characterization of aflatoxin biosynthesis genes of aspergillus flavus from peanuts production area. Legume Research. 42(5): 609-614. doi: 10.18805/LR-508.

Leisova, L., Kucera, L., Minarikova, V. and Ovesna, J., (2005). AFLP-based PCR markers that differentiate spot and net form of *Pyrenophora teres*. Plant Pathology. 54: 66-74.

McDonald, M.R., Jaime, M.A. and Hovius, M.H.Y., (2004). Management of diseases of onion and garlic. In Diseases of fruits and vegetables. Eds. Naqvi SAMH. Kluwer Academic Publishers. The Netherlands. 149-200.

Prajapati, B.K., Patil, R.K., Alka, Chavda, H.C. (2016). Molecular characterization of *Aspergillus niger* isolates inciting black mould rot of onion through RAPD. International Journal of Agriculture, Environment and Biotechnology. 9: 637-642.

Rani, D., Sudini, H., Reddy, P., Mangala, U., Kumar, K., (2018). Pathogenic and molecular variability of *Aspergillus niger* Isolates causing collar rot disease in groundnut, Int. J. Pure App. Biosci. 6: 840-848.

Samson, R.A., Jos, A.M.P., Houbraken, J.A.M.P., Kuijpers, A.F. A., Frank, J.M. and Frisvad, J.C., (2004). New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri.* Studies in Mycology. 50: 45-61.

Schuster, E., Dunn-Coleman, N., Frisvad, J.C. and Van Dijck, P.W.M (2002). On the safety of *Aspergillus niger* -A review. Applied Microbiology and Biotechnology. 59: 426-435.

Sharma, R. (2012). Pathogenicity of *Aspergillus niger* in plants. Cibtech Journal of Microbiology. 1: 47-51.

Singh, R., Rai, T.S., Sharma, N.S., Arora, A.K., Kaur, Paviter (2017). Evaluation of a real time polymerase chain reaction assay for the detectionof aflatoxin/sterigmatocystin producing strains of *Aspergillus* spp. Indian Journal of Animal Research. 51(4): 676-678. doi: 10.18805/ijar.v0iOF.7263.

Tawfik, E., Alqurashi, M., Aloufi, S., Alyamani, A., Baz, L., Fayad, E. (2022). Characterization of mutant *Aspergillus niger* and the Impact on certain plants. Sustainability. 14:1936. https://doi.org/10.3390/su14031936.

Tysony, J.L. and Fullerton, R.A., (2004). Effect of soil borne inoculums on incidence of onion black mould (*Aspergillus niger*). New Zealand Pl. Prot. 57: 138-141.

Welsh, J. and McClelland, M. (1990). Finger printing genomes using PCR with arbitrary primers. Nucleic Acids Res. 18: 7213-7218.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalskiand, J.A. and Tingey, S.V., (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.

Yuan, G.F., Liu, C.S. and Chen, C.C. (1995). Differentiation of *Aspergillus parasiticus* from *Aspergillus sojae* by random amplification of polymorphic DNA. Appl. Environ. Microbiol. 61: 2384.