The Bioscan 11(4): 2105-2110, 2016
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

ASSESSMENT OF FUNGI ASSOCIATED WITH BLACK POINT DISEASE OF WHEAT AND GENETIC VARIATION AMONGST THE MOST DOMINANTLY ISOLATED FUNGUS [*ALTERNARIA ALTERNATA* (FR.) KESSL.]

D. J. PATEL*[1] AND D. B. MINIPARA[2]
[1]Department of Plant Pathology,
[2]Centre of Excellence in Biotechnology
B. A. College of Agriculture, Anand Agricultural University, Anand-388110. INDIA.
e-mail: address:aau.dipen@yahoo.com

KEYWORDS
RAPD
Black point disease
Lternariaalternata
Isolates
Band

Received on :
13.11..2015

Accepted on :
27.10.2016

***Corresponding author**

ABSTRACT

The black point is economically crucial disease that conveys severe qualitative loss to wheat farmers globally. The present investigation included popularly cultivated wheat varieties namely GW 496, GW 366 and Lok 1,sampled from wheat growing pockets of middle Gujarat region of India. The standard blotter isolation technique detected eight fungal species belonging to diverse genera. Out of them,*Alternariaalternata* found predominantly associated with 46.83% of frequency.The RAPD analysis of the seven isolates of *Alternariaalternata*, corresponding different regions of the middle Gujarat, analyzed by 15 random primers, produced 253 loci. Out of which, 238 loci were polymorphic. Total number of 599 bands were produced by selected random primers. The primers revealed 92.90% mean polymorphism. More over, average numbers of polymorphic loci obtained per primer (Assay Efficiency Index) were 15.86. The average similarity coefficient observed was 0.391 and average PIC value was 0.89. The Dendrogram of pooled molecular data divided seven isolates into two clusters indicating existence of variability among the isolates. The isolates "DEL" and "KEL" demonstrated significantly the highest genetic variations.

INTRODUCTION

Black point disease was first reported in the United States in 1913 (Watkins and Prentise, 1997). Afterwards, Dastur (1932) reported its occurrence first time in Northern parts of India. The infection is generally observed in South-West and North-Eastern states of India (Karwasra *et al.*, 2006). The disease is a cause of multiple pathogens (Sisterna and Sarandon, 2005). Several fungi belonging to various genera such as *Alternaria alternata, Bipolaris sorokiniana,Cochliobolus sativus,Clado sporium clado sporioides, Curvulari alunata,Curvularia pallescens,Drechslera halodes,Fusariumgr aminarium and F. culmorum* are found frequently associated to black pointed wheat seeds(Hudec and Muchova, 2008). *Alternaria alternata* could be isolated with 62-90% frequency from infected seeds (Khulbe *et al.*, 2011).Other seed-borne fungi such as *Fusarium moniliforme* (52%), *Drechslera Sp.* (26%), *Curvularia lunata* (28%), *Cladosporium sp.* (15%) and *Penicillium spp.* (10%) are isolated with respective frequency from wheat seeds showing black point symptoms (Pathak and Zaidi, 2013).However, *Alternaria alternata* is considered as the predominant one (Zuo *et al.*, 2011). The epidemiology of the black point disease is poorly understood under middle Gujarat conditions. A strategic investigation to determine the causal pathogen(s) would help researchers to focus precisely on the development of management practices. Moreover, the molecular techniques have provided abundant genetic markers that can be used to assess the genetic structure of field populations of plant pathogens.Molecular technique such as Random Amplified Polymorphic DNA- Polymerase Chain Reaction (RAPD) has been used to study variability of Genus *Alternaria* in different parts of the world (Marak *et al.*, 2014).The genetic variation amongst dominant pathogenic isolates would be helpful to understand the developing strains of pathogen that will assist researchers to understand virulence of biotic cause(s) and develop the management practices accordingly. It would contribute a preliminary idea to develop an effective programme of breeding disease resistance cultivars. Pragmatically, the pathogen(s) isolated from black pointed wheat seeds are poorly understood at molecular level. Considering above facts, the present investigation was planned to determine the fungi associated with the disease and to characterize the frequently isolated pathogen by RAPD technique.

MATERIALS AND METHODS

Sample collection

Fresh seeds of popularly cultivated wheat varieties preferably *viz.*, GW 366, GW 496,Lok 1 and GW 1 were collected after harvesting season form the farmers' fields of wheat growing areas of middle Gujarat districts *viz.*, Anand, Ahmedabad,

Kheda and Vadodara. Atleast 20 samples were collected from each of the districts. From the harvested seed lot five primary samples were collected and mixed to have composite samples. Working samples were prepared from composite samples by repeated halving method (Neergard, 1968).

Assessment of fungal association

Black point infected seeds were treated with 0.5 per cent sodium hypochlorite solution for one min by standard blotter and standard agar plate method (Bhale *et al.,* 2001). Total 400 seeds from each of the seed samples were assessed. Fungal colonies developed on each of the seeds, after incubation at 25 ºC for about seven days, were critically examined by hand lens and then under microscope. The association frequency of each of the fungi was recorded accordingly. Isolated fungi, after purification by single spore isolation or hyphal tip method, were maintained on potato dextrose agar (PDA) at low temperature (5 ºC).

Identification of Pathogen

The most frequently isolated fungal cultures were sent to Indian Type Culture Collection (ITCC), New Delhi for identification. Each of them were identified as *Alternaria alternata*.In terms of molecular study, isolates of *Alternaria alternata* were isolated from samples collected from various locations *viz*., Dethli, Thasra, Arnej, Anand, Sankarda, Menthal and Kelanpur. Each of the seven isolates used in study was coded as DTH, THS, ARN,AND, SAN, MTL and KEL representing the area respectively. The codes were used in study to easily identify the isolates.

Genomic DNA isolation

The fungus was grown on potato dextrose broth (PDB) by shaking for 5-7 days at 26 º C. Mycelium was collected by filtration through sterilized filter paper lining a Buchner funnel and, then extensively washed with water. The mycelium was blotted dry between the layers of tissue and immediately frozen in liquid nitrogen in foil packets. The tissue was gently broken into fine pieces by crushing the foil envelops with a pestle and mortar. Care was taken not to allow the tissue to thaw as this causes lyses and the release of endogenous nuclease. Extraction of DNA from mycelia was done using Cetyl-Trimethyl Ammonium Bromide (CTAB) method (Niu *et al.*,2008).

PCR amplification using RAPD primers

A total of 40 decamer primers belonging to OPA and OPE series were screened for RAPD analysis. Of these, 15 primers were selected based on repeatability. The RAPD primers used for the analysis of random amplification of polymorphic DNA to study the polymorphism present in the isolates of *Alternaria alternata* are mentioned in Table 1. PCR was carried out in 25ìl reaction volumes containing 2.5 ìl of 10 x Taq assay buffer (Tris with15mM MgCl2), 10 mM of each dATP, dCTP, dGTP and dTTP, 5U Taq polymerase (BioLabs, UK), 10picomole of primer and approx. 50ng of template DNA (Niu *et al.,* 2008).Amplification were carried out in a thermo-cycler (Applied BiosystemVeriti, CA, USA) programmed for 40 cycles with an initial denaturation at 94ºC for 4 min followed by cycling conditions of denaturation at 94º C for 1 min, annealing at 1min at 38°C and extension at 72º C for 2 min. After 40 cycles, there was a final extension step of 7 min at 72º C. The amplicons were analyzed on 1.6% agarose gels and detected by staining with ethidium bromide. UV trans-illuminated gels were photographed with gel documentation system.

Data scoring and analysis

Data was scored on the basis of presence or absence for analysis. The scores 1 and 0 indicates the presence or absence of bands respectively. The data were maintained in the excel sheet format for further analysis. The polymorphism percentage was calculated as per the method suggested by Blair *et al.* (1999). The data generated by RAPD were analyzed with the software POPGENE 32 version 1.31 (Yeh and Boyle 1997). Diploid data analysis for dominant marker was performed with the assumption of Hardy-Weinberg equilibrium. Multiple populations were used for the estimation of effective allele number, polymorphic loci, Nei's unbiased measures of genetic identity and genetic distance as well as for dendrogram construction. A dendrogram was drawn based on Nei's (1978) genetic distances using UPGMA. This program is an adoption of program NEIGHBOR of PHYLIP version 3.5c by Joe F elsenstein. The drawing was executed for multiple populations.

RESULTS

Association of fungi

The investigation revealed eight fungi belonging to diverse genera *viz., Drechslera rostrata, Alternaria alternata, Drechslera* sp.,*Curvularia lunata,Cladosporium clado sporiodes, Fusariumgr aminarium, Fusarium* sp. and *Cheatomium* sp. with association frequency ranging from 3.16% to 46.83%. *Alternaria alternata* was found the most dominantly associated to black point infected seeds of all the three varieties (Table: 2). The precise data analysisrevealed 40.25%, 52.50% and 47.75% frequency of association with GW 496, GW 366 and Lok 1 varieties, respectively. Next dominantly associated fungus was *Drechslera rostrata* showing the frequency of 18.25%, 22.5% and 16.75% with GW 496, GW 366 and Lok 1 varieties, respectively, followed by *Drechslera* sp., which showed 14.50%, 18.75% and 16.25% frequency with GW 496, GW 366 and Lok 1 variety, respectively.Thus, *Alternaria alternata, Drechslera rostrata* and

Table 1: Sequences of primers used for the RAPD analysis

Sr. No.	Primer[a]	Sequence (5'-3')	Bases
1	OPA 1	CAG GCC CTT C	10
2	OPA 7	GAA ACG GGT G	10
3	OPA 11	CAA TCG CCG T	10
4	OPE 3	CCA GAT GCA C	10
5	OPE 4	GTG ACA TGC C	10
6	OPE 5	TCA GGG AGG T	10
7	OPE 6	AAG ACC CCT C	10
8	OPE 7	AGA TGC AGC C	10
9	OPE 8	TCA CCA CGG T	10
10	OPE 9	CTT CAC CCG A	10
11	OPE 11	GAG TCT CAG G	10
12	OPE 14	TGC GGC TGA G	10
13	OPE 15	ACG CAC AAC C	10
14	OPE 16	GGT GAC TGT G	10
15	OPE 17	CTA CTG CCG T	10
Total			1500

[a]OPE and OPA are standard series of primers selected for study.

Table 2 : Fungal association with black point infected wheat seeds

Fungus	Frequency (%) of fungal association Wheat varieties			Mean
	GW 496	GW 366	Lok 1	
Drechslera rostrata	18.25	22.50	16.75	19.16
Alternaria alternata	40.25	52.50	47.75	46.83
Drechslera sp.	14.50	18.75	16.25	16.50
Curvulari alunata	8.00	10.25	9.50	9.25
Cladosporium cladosporiodes	3.25	3.75	2.50	3.16
Fusarium graminarium	11.25	13.75	12.25	12.41
Fusarium sp.	10.25	12.50	12.00	11.58
Chaetomium sp.	2.75	4.00	3.75	3.50
Mean	13.56	17.25	15.09	

Table 3 : Assay of DNA from the *A. alternata* isolates obtained through nanodrop technique

Isolates[a]	Area[b]	(260/280) Wavelength ratio	Concentration (ng/μl)
DTH	Dethli	2.07	1861.4
THS	Thasra	2.08	1300.5
ARN	Arnej	2.12	1677.3
AND	Anand	2.12	2491.6
SAN	Sankarda	2.06	1875.7
MTL	Menthal	2.13	2108.1
KEL	Kelanpur	2.11	653.4

[a] Identification codes of isolates of *Alternaria alternata*;[b]Geographic locations of the isolates collected in India

Table 4 : Results of RAPD analysis

Sr. No.	Primer	Molecular weight range (bp)[b]	No. of bands	No. of loci	No. of polymorphic loci	Polymorphism (%)	PIC[a] value
1	OPA 1	365-1854	14	04	03	75.00	0.61
2	OPA 7	252-1353	83	19	13	68.42	0.93
3	OPA 11	262-1444	60	18	15	83.33	0.92
4	OPE 3	287-1445	35	19	19	100.0	0.92
5	OPE 4	259-1554	48	21	19	90.47	0.92
6	OPE 5	145-1342	41	21	21	100.0	0.93
7	OPE 6	185-918	24	11	11	100.0	0.88
8	OPE 7	298-1040	43	17	16	94.11	0.90
9	OPE 8	252-2739	30	10	09	90.00	0.85
10	OPE 9	308-1380	49	20	20	100.0	0.93
11	OPE 11	213-2085	40	22	22	100.0	0.94
12	OPE 14	334-1952	29	18	18	100.0	0.92
13	OPE 15	285-1938	31	21	21	100.0	0.94
14	OPE 16	640-1353	37	19	19	100.0	0.92
15	OPE 17	369-1052	35	13	12	92.30	0.88
Total			599	253	238	-	-
Average			39.93	16.86	15.86	92.90	0.89

[a]PIC = Polymorphism Information Content; [b]Bp = Base Pair

Table 5: Genetic similarity matrix of pooled RAPD data based on Jacard's similarity coefficient

	DTH	THS	ARN	AND	SAN	MTL	KEL
DTH	1						
THS	0.216	1					
ARN	0.210	0.267	1				
AND	0.172	0.171	0.286	1			
SAN	0.080	0.872	0.112	0.186	1		
MTL	0.069	0.102	0.078	0.145	0.254	1	
KEL	0.054	0.086	0.155	0.092	0.178	0.165	1

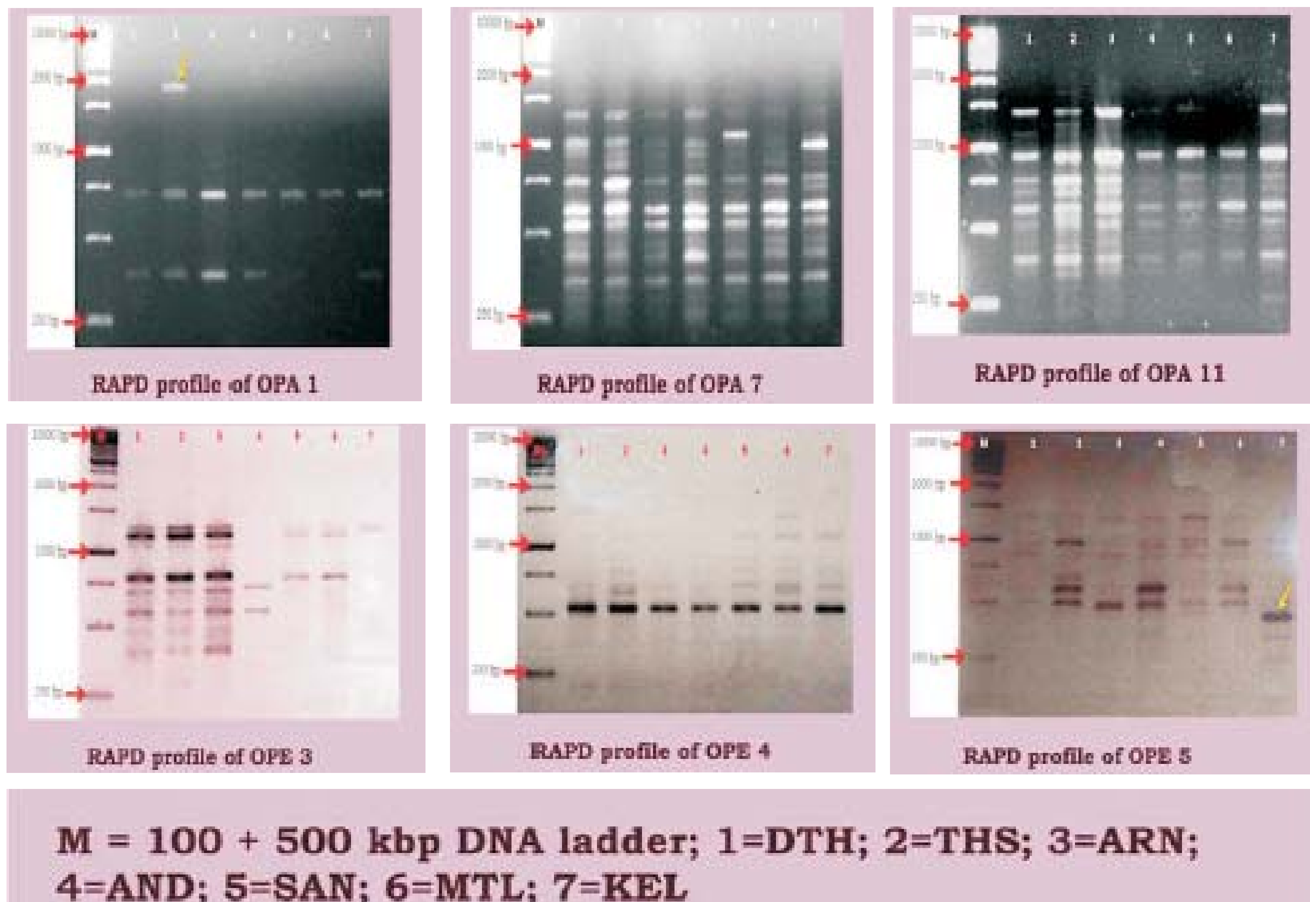
DTH = Dethli, THS = Thasra, ARN = Arnej, AND = Anand, SAN = Sanand, MTL = Menthal, KEL = Kelanpur

Drechslera sp. were predominantly found with black point infected seeds. Frequency of association *Fusariumgr aminarium, Fusarium* sp. and *Culvul arialunat* aranged between 8.00% to 13.75%. Remaining two fungi *viz.,Clado sporiumcladosporiodes* and *Cheatomium* sp. revealed quite negligible association *i.e.* 2.50% to 4.00%.

Molecular characterization

Qualitative and quantitative analysis of genomic DNA

The concentration of DNA was obtained between 653.4 to 2491.6 ng/ìl and A260/A280 was 2.06 to 2.13. None of the DNA samples analyzed were of poor quality (Table 3).



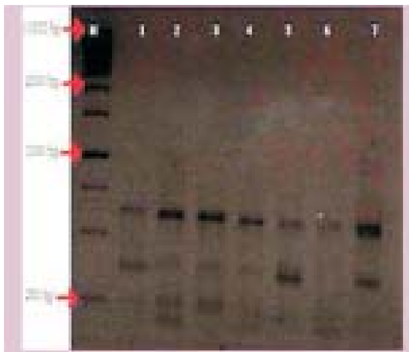
RAPD analysis

The data collected from random amplification of polymorphic DNA with 15 arbitrary primers (Table: 4) produced 253 total loci and the total bands produced were 599. Out of the 253 loci produced, 238 were polymorphic and hence the total polymorphism percentage was found to be 92.99 %. The average PIC (Polymorphism Information content) values for RAPD was 0.89. Average number of polymorphic loci obtained per primer (Assay Efficiency Index) was found to be 15.86. The molecular size of the amplified PCR products ranged from 145 bp to 2739 bp. The RAPD marker OPA 7 produced maximum number of 83 bands, while OPA 1 produced the minimum number of 14 bands.As much as 8 primers out of the selected 15 primers *viz*., OPE 5, OPE 6, OPE 8, OPE 9, OPE 11, OPE 14, OPE 15 and OPE 16 showed 100 per cent polymorphism (Fig 1). The lowest polymorphism (68.42 %) was found in the primer OPA 7. The highest PIC value obtained were 0.94 for OPE 11 and OPE 13 markers and lowest PIC value was 0.61 for OPA 1. The highest similarity index value of 0.872 was observed between SAN and THS isolates. Simultaneously, the least similarity index value of 0.054 was found between KEL and DTH isolates. The average similarity coefficient among isolates was 0.391 (Table: 5). Specific band observed in THS isolate of Kheda district was of 1853 bp. Moreover, Specific bands were observed in primer OPE 1 and OPE 5,7,11.

Dendrogram (Fig 2) based on "Nei's (1978) unbiased measures of genetic distance (Table5) by Unweighted Pair Group Method with Arithmetic Average (UPGMA) method" formed two clusters namely A and B. The cluster A consisted DTH, THS, ARN and AND isolates Simultaneously, the cluster B consisted of SAN, MTL and KEL isolates. Cluster A was further divided into two clusters namely A1 and A2. The cluster A1 included DTH isolate. Moreover, cluster A2 was sub divided in to two clusters *viz.*, A2a and A2b. The cluster A2a consisted THS isolate. However, the cluster A2b was further divided into two sub clusters namely A2ba and A2bb which included ARN and AND isolates, respectively. Simultaneously, the cluster B was divided into two clusters namely B1 and B2. Moreover, cluster B1 was fragmented in to two sub clusters *viz.,* B1a and B1b which consisted SAN and MTL isolates, respectively. The cluster B2 included KEL isolate.

DISCUSSION

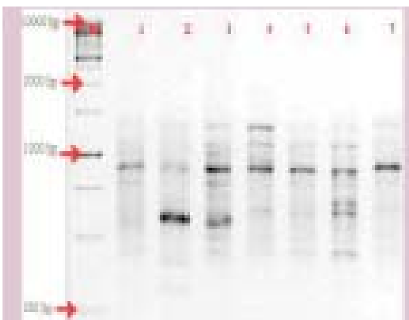
Overall, the mean frequency of fungal association irrespective of specific fungus was the maximum with variety GW 366 (13.25 %) as compared to Lok 1 (11.56 %) and GW 496 (10.47 %). In was quite interesting to note that variety GW 366 revealed significantly the highest infection. The seeds of GW 366 are bolder than Lok1 and GW 496 varieties. Flowers of bolder variety remain open longer than the timid varieties.



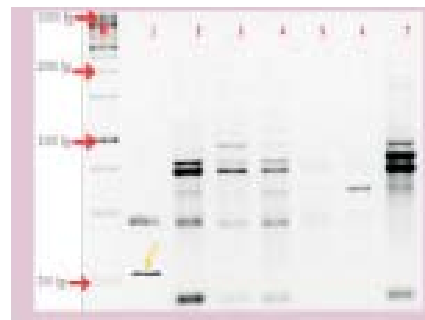
RAPD profile of OPE 6

RAPD profile of OPE 7

RAPD profile of OPE 8



RAPD profile of OPE 9

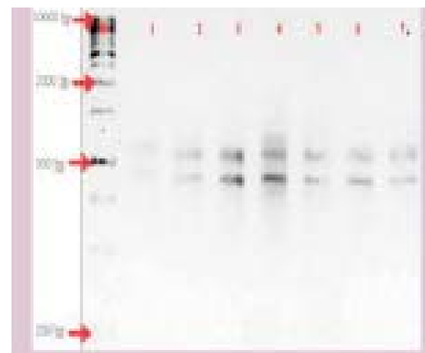


RAPD profile of OPE 11

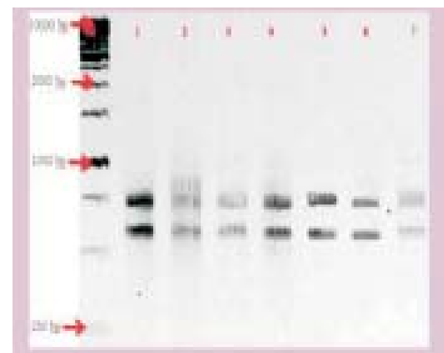
RAPD profile of OPE 14

M = 100 + 500 kbp DNA ladder; 1=DTH; 2=THS; 3=ARN; 4=AND; 5=SAN; 6=MTL; 7=KEL

RAPD profile of OPE 15



RAPD profile of OPE 16



RAPD profile of OPE 17

M = 100 + 500 kbp DNA ladder; 1=DTH; 2=THS; 3=ARN; 4=AND; 5=SAN; 6=MTL; 7=KEL

The open florets increase the chances for pathogenic penetration that cause the black point symptoms to the developing seed under field conditions. On the basis of present investigation, *A. alternata* may be attributed to one of the prime causes of black point of wheat which tallies with the earlier report of Khulbe *et al*. (2011); Bhoyar *et al*. (2014) and Srivastava *et al*. (2014).

It is crucial in plant protection to understand genetic structure of pathogens for better understanding their virulence. Moreover, the pathogenic resistance is enhancing in agro-ecosystem because of environmental changes like crop rotation, tolerant varieties, fungicides and irrigation (Kakvan *et al.,* 2012). In such circumstances, the molecular characterization of pathogens is an efficacious way to discover

virulence of pathogenic isolates which would help researchers to manage the disease expeditiously. In this study, 15 isolates of *Alternaria alternata,* isolated from wheat seeds were attempted by RAPD technique for molecular characterization.The results are corroborated with (Zuo *et al.*) 2011 who characterised *Alternaria Spp.* isolated from black point disease by 17 random RAPD primer,that produced 151 amplicons with 100%polymorphisms.The present investigation revealed significant genetic diversity amongst the pathotypes. Interestingly, the molecular variation most commonly observed in plant pathogenic fungi is generally due to mutation, gene flows and recombination. Constantly interactions between host and pathogen might causeshigh diversity in pathogen (Tack *et al.,* 2012).Present study reveals significantly the highest variation amongst two isolates "DTH" and "KEL". Hence, it might have caused due to variation mating patterns, gene flowsand mutation. More over, variation in climatic conditions between two regions might have added genetic diversity.On contrary to that, "AND" and "ARN" isolates had similar climatic conditions which expressed significantly the least genetic variation. The results tallies with Mann *et al.*(2014) who reported that the climatic difference among the geographical areas, from where the isolates belongs to, play significant role in genetic modifications of pathotypes.

In conclusion, the disease is the cause of multiple pathogen. However, *Alternaria alternaria* was revealed as the prime cause of the disease. Molecular characterization of *Alternaria alternata*, carried out by 15 arbitrary RAPD primers produced 253 amplicons and 599 bands. As much as 238 amplicons were found polymorphic with on an average of 92.90 per cent polymorphism. The average 0.89 Polymorphic Information Content (PIC) value was obtained. Dendrogram generated by pooled molecular data of 15 RAPD primers formed two clusters namely A and B. The cluster A included isolates DTH, THS, ARN and AND, while the cluster B included isolates SAN, MTL and KEL. Overall, the molecular weight of the amplicons ranged between 145 to 2739 bp. Variations in reproduction, host-pathogen interactions and climatic factors generated genetic diversity amongst the isolates.

ACKNOWLEDGEMENT

Authors are grateful to Dr. S. T. Patel, retired scientist (STR), Department of Plant Pathology, B.A. College of Agriculture, Anand Agricultural University, Anand for his scientific guidelines and support.

REFERENCES

Bhale, M., Khare, D., Rawt, N. and Singh, D. 2001. Seed borne diseases objectionable in seed production and their management. Scientific Publishers, Jodhpur, India.

Bhoyar, P., Chandankar, V., Bagde, V. ,Borkar, D and Sonone, J. 2014. Studies on seed borne mycoflora and effect of bioagents and fungicides on wheat seed health. *The Bioscan*.**9:** 1285-1289.

Blair, M., Panaud, O. and McCouch, S. 1999. Inter simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and finger printing in rice (*Oryza sativa* L.). *Theor. Appl. Genet.***98:** 780-792.

Dastur F. 1932. Foot rot and black point diseases of wheat in central provinces. *Agri. Livestock in India.***2:** 275-282.

Hudec, K. and Muchova, D. 2008. Correlation between black point symptoms and fungal infestation and seedling viability of wheat kernels. *Plant Protect. Sci.* **44:** 138-146.

Kakvan, N., Hamidreza, Z., Bahar, M., Hossein, T. and Shahab, H. 2012. Library Study on pathogenic and genetic diversity of *Alternariaalternata* isolated from citrus hybrids of Iran, based on RAPD-PCR technique. *Euro. J. of Exp. Biol.***2:** 570-576.

Karwasra, S., Beniwal, M. and Beniwal, R. 2006. The occurrence and fungi associated with black point of wheat in Haryana. *Crop Res.* **32:** 118-120.

Khulbe, D., Srinivas, P. and Agarwal, V. 2011. Diversity of black point symptoms and associated fungi in wheat and seed quality. *Ann. of Pl. Prot. Sci.***19:** 383-387.

Mann, M.,Spadari,C.,Feltrin,T.,Paula, A.,Frazzon,.G., Carlos, J. and Van Der Sand, S.2014. Genetic variability of *Bipolaris sorokiniana*isolates using URP-PCR. *Trop. Plant Pathol.* **39:** 163-171.

Marak, T., Ambesh, B. and Das, S. 2014. Cultural, morphological and biochemical variations of *AlternariaSolani* causing diseases on Solanaceous crops. *The Bioscan*. **9:** 1295-1300.

Neergard, P.1968. Seed-borne diseases and Indian agriculture. Danish Government Institute of Seed Pathology for Developing countries, Copenhagen, Denmark, pp.224-237.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics.***89:**583-590.

Niu, C., Kebede, H., Auld, L., Woodward, J., Gloria, B. and Wright, R. 2008. A safe and expensive method to isolate high quality plant and fungal DNA in an open laboratory environment. *African J. Biotechnology*.**7:** 2818-2822.

Pathak, N. and Zaidi, R. 2013. Fungi associated with wheat seed discolouration and abnormalities in in-vitro study. *Agricultural Sciences*.**9:** 516-520.

Sisterna, M. and Sarandon, S. 2005. Preliminary studies on the natural incidence of wheat black point under different fertilization levels and tillage systems in Argentina. *Plant Pathology J.***4:** 26-28.

Srivastava, J., Kushwaha, G. and Shukla, D. 2014. Black point disease of wheat and its implications on seed quality. *Crop Res.***4:** 21-23.

Tack, A., Thrall, P. Barrett, L., Burdon, J. and Laine. A. 2012. Variation in infectivity and aggressiveness in space and time in wild host-pathogen systems – causes and consequences. *J. Evol. Biol.* **25:** 1918-1936.

Toklu, F., Davut, S., Mehmet, B. and Tolga, K. 2008. The relationship between black point and fungi species and effects of black point on seed germination properties in bread wheat. *Turk. J. Agric.* **32:** 267-272.

Watkins, J. and Prentise L. 1997. Black point disease of wheat. Neb Guide. Publ. Institute of Agriculture and Natural Resources, Lincoln Extn.,Univ of Nebraska, U.S.A.

Yeh, F. and Boyle, T. 1997. Population genetic analysis of codominant and dominant markers and quantitative traits. *Belgian J. Botany*.**129:**157.

Zuo, R., Yuan, H., Xing, X., Zhang, M.,Dai, J., Yu,Q. and Li, H. 2011. RDNA-ITS sequence analysis of the dominant pathogens of wheat black point *Alternaria spp.*in Henan and their Similar Species.*J. of Agri. Biotechnol.* **3:***68-74.*