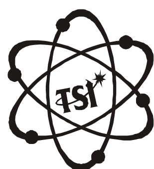
ISSN : 0974 - 7435 Volume 7 Issue 2



Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 7(2), 2013 [49-53]

Molecular characterization of *Alternaria alternata* (Apple pathotype) from Kashmir valley

Yash Paul Khajuria[1, 2*], Sanjana Koul[1], M.K.Dhar[1]

[1]School of Biotechnology, University of Jammu, Jammu and Kashmir, Jammu-18006, (INDIA)

[2]National Institute of Plant Genome Research, New Delhi-110067, (INDIA)

E-mail : yashkhajuria@gmail.com

ABSTRACT

Apple is one of the most important economically fruit plants of the world. *Alternaria mali* (*Alternaria alternata* apple pathotype) causes huge loss by infecting leaves and fruits. It can infect upto 85% of leaves in highly infected orchards. The fungus causes leaf spots which enlarge in zonate, circular or cresent shaped rings. Understanding the genetic variability of the pathogen is very important for devising the strategies to control it. DNA based molecular markers have been successfully used for studying the genetic variation in different fungal pathogens. During present investigation samples were collected from different areas of Kashmir valley. For molecular characterization two molecular markers RAPD and ISSR were used for characterization. Clustering in dendrogram indicates that isolates from all regions were intermixed. Also there were no cultivar specific isolates identified. © 2013 Trade Science Inc. - INDIA

KEYWORDS

Kashmir valley;
Alternaia blotch;
Alternaria alternata (Apple pathotype);
Molecular characterization;
RAPD;
ISSR.

INTRODUCTION

Apple (*Malus* × *domestica*) is main fruit tree species of the moderate zone. A large number of fungal pathogens attack apple. Important fungal diseases of apple are apple scab, powdery mildew, *Alternaria* blotch etc. Alternaria blotch is caused by *Alternaria mali* which belongs to the *A. alternata* (E. M. Fries) Keissler group. Different pathogenic species of *Alternaria* cannot be distinguished from one another either morphologically or using molecular markers and are named as *A. alternata* pathotypes. Therefore, *A. mali* is more appropriately known as *A. alternata* apple pathotype. It infects mainly leaves (up to 50 to 60 %) but sometimes fruits may also get infected. On fruit, the disease is characterized by the development of small necrotic spots. Disease spreads by means of conidia and its spread is particularly favoured by rainfall and high temperature[15]. Morphologically spores have false beak and have both transverse and longitudinal septa and size falls in range of 20.6 × 9.25 µM[5,17].

Apple pathotype acts on susceptible cultivars by producing specific toxin known as AM toxin. This toxin is present on the small extra chromosome of 1.8Mb[14]. Most of commercial cultivars are generally sensitive to AM toxin. Because Apple blotch is wide spread and has the potential to spread new areas, it is essential to characterize the fungus. Also, understanding the genetic

structure of pathogen can be helpful in devising strategies for controlling the disease. Several studies have highlighted the importance of molecular markers in genetic studies of fungi[16]. Using molecular markers like RAPD and ISSR. RAPD has been proved to be an efficient technique for characterization of *Alternaria* species and has been used for many phytopathogenic fungi. ISSR based characterization is more reliable because of use sub arbitrary primers. There are no reports of molecular characterization *Alternaria alternata* from Kashmir valley. Our objective was to detect genetic variation among different isolates of *Alternaria alternata* (Apple pathotype) using ISSR and RAPD techniques.

EXPERIMENTAL

Collection of infected material

Infected leaf samples were collected from the orchards at different time intervals during 2003 to 2007 from different areas of valley. After collection, the leaves were placed between the folds of the newspapers for drying. Efforts were made to remove as much moisture as possible, in order to avoid cross contamination by other fungi. The samples were kept in the paper bags and were marked indicating different accession numbers. The bags were wrapped in polythene and kept at 4°C to minimize the degradation and to prevent the contamination.

Culturing, purification and preservation of fungal isolates

Potato dextrose agar medium (PDA) is the basic medium for growth of many fungi. Other media tried for the culture of the fungi are apple infusion agar (AIA) and corn meal agar (CMA). The PDA medium worked very well for apple pathotype. The antibiotic chloramphenicol (50 µg/ml) was added to the medium to avoid bacterial contamination.

Leaves were treated with disinfectants to eliminate the bacteria and other non–fixed saprophytes from the surface of the leaf. Leaves were first washed with autoclaved distilled water, dipped in 70% ethanol for 10 seconds, and again washed using autoclaved distilled water. Small pieces from the infected area of the leaf were cut and washed in sterile distilled water. The leaf bits were inoculated on PDA slants and kept at 25°C. The fungal growth was observed after 10–12 days of inoculation. The isolates obtained from leaves were again sub–cultured to obtain pure cultures. Out of several isolates obtained, finally 20 isolates were selected for further studies (TABLE 1). Single spores were obtained on water agar medium. For long term storage, spores were mixed in 10% glycerol in preservation vials and stored at ultra low temperature (–80°C). These cultures were stored as stock cultures for future use.

TABLE 1 : Name and location of the orchards surveyed

Sr. No.	Orchard No.	Name of the Orchard	Area (District)	Division
1.	1	M.Siddique Orchard	Dhogam (Pulwama)	Kashmir
2.	3	Karam Bibi Orchard	Chattergam (Budgam)	Kashmir
3.	4	Rehmatullah Orchard	Kralpathri (Budgam)	Kashmir
4.	6	Kachdoora Orchard I	Shopian (Pulwama)	Kashmir
5.	7	Kachdoora Orchard II	Shopian (Shopian)	Kashmir
6.	8	Muzzafar Orchard	Kakpura (Pulwama)	Kashmir
7.	10	Dhar Orchard	Zakura (Ganderbal)	Kashmir
8.	11	Dee Orchard	Indergam (Baramulla)	Kashmir
9.	12	Sikh Orchard	Nowpora (Baramulla)	Kashmir
10.	15	Govt. Horticulture Orchard	Zengam (Baramulla)	Kashmir
11.	16	Govt. Horticulture Orchard	Zanapora (Shopian)	Kashmir
12.	17	Kli Orchard	Aharbal (Shopian)	Kashmir
13.	18	Wani Orchard	Aharbal (Shopian)	Kashmir
14.	19	Rehman Orchard	Batote (Doda)	Jammu
15.	20	Imitiaz Orchard	Batote (Doda)	Jammu

Fungal DNA isolation

Genomic DNA was extracted using the CTAB method with minor modifications (Doyle and Doyle, 1990). After 12–15 days of inoculation, hyphal mass was obtained from flasks by filtering the broth through sterile muslin cloth. Fungal mass was then dried between the folds of sterile filter paper. It was powdered in a pre–cooled pestle and mortar using liquid nitrogen. About 1gm of fungal powder was added to preheated 10 ml CTAB buffer in centrifuge cups and incubated for 30 minutes at 65°C in a water bath. Samples were mixed every 10 min by inverting centrifuge cups 5–6 times to make sure that the tissue mixed well with the buffer. 10 ml chloroform: isoamyl alcohol (25:1) was added to each tube. After proper mixing, the samples were centrifuged to sediment the cell debris and the upper aqueous phase was transferred to another tube.

Later, equal volume of chilled DNA grade ethanol was added to precipitate DNA. Samples were mixed gently and centrifuged at 8000 rpm for 5 minutes. Supernatant was discarded and pellets were washed 3 times with 70% ethanol, air dried and dissolved in 100 µl TE. The DNA samples were treated with 1µl RNase (10 mg/ml) for three hours at 37 °C to remove RNA.

TABLE 2 : Pure cultures of *Alternaria* alternata isolated on PDA slants

S. No.	Accession no.	Source cultivar
1.	4-57.3	Treil Kicham
2.	6-Acc l.1	Unidentified
3.	6-12.2	American Treil
4.	10-12.1	American Treil
5.	6-15.2	Chemura
6.	10.15.3	Chemura
7.	6-13.0	Balpuri Kashmiri
8.	6-77.0	Gole French
9.	6-82.2	Gulshan Anar
10.	7-7.1	Ambri (Shopian)
11.	7-11.0	Ambri (Jungli)
12.	7-18.0	Dilruba
13.	7-23.3	Red Delicious
14.	7-23.5	Red Delicious
15.	7-37.1	Maharaji
16.	7-41.2	Pokhla
17.	7-41.3	Pokhla
18.	7-55.1	White Cider
19.	6-62.2	Lal Farashi
20.	7-75.0	New Pholka
21.	7-Acc l05	Unidentified
22.	8-30.3	Hazratbali
23.	10-9.2	Ambri (Vilayti)
24.	10.N.2	Unidentified
25.	10-21.0	French Lal
26.	10-24.2	Royal Delicious
27.	11-24.1	Royal Delicious
28.	10-45.3	Razakwari
29.	11.89.0	Chita Titto
30.	Alt	Unidentified

The RNA free DNA was further purified by extracting with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1) followed by centrifugation at 5000g for 10 min. The aqueous phase was collected and mixed with equal volume of chloroform followed by centrifugation at 5000 g for 10 min. The DNA was precipitated from the aqueous phase using chilled ethanol. The DNA was obtained as pellet after centrifugation which was dissolved in TE and stored at –20°C.

Quantification of DNA was done both spectrophotometrically as well as by agarose gel electrophoresis using lambda DNA as standard. In the latter case, band intensity of sample DNA was compared with the band intensity of lambda DNA of various concentrations and the concentration of sample DNA was thus determined.

RAPD (Random Amplified Polymorphic DNA) analysis

PCR was performed in 25 µl volume containing 3 mM $MgCl_2$, 1 × *Taq* polymerase buffer, 200 µM of dNTPs, 0.8 µM primer, 0.4 units of *Taq* polymerase and 50 ng of DNA. The thermal cycling conditions consisted of an initial denaturation for 5 minutes at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 36°C and extension for 2 min at 72°C with a final extension of 10 min at 72°C.

Samples were visualized on 1.4% agarose gel. The gels were scored for the presence and absence of bands. 25 different RAPD primers were used in present study and 8 primers showing best polymorphism were selected for further analysis.

ISSR (Inter Simple Sequence Repeats) analysis

Amplification was performed in a 25 µl reaction volume which contained 1 X *Taq* buffer, 3 mM $MgCl_2$, 200 µM of each dNTPs, 0.5 µM of primer, 10–30 ng template DNA, and 0.6 units of *Taq* DNA polymerase. The thermal cycler conditions consisted of initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation for 30s at 94°C, annealing at 45°C for 30s and extension for 2 min at 72°C, with the final extension of 10 minutes. 28 ISSR primers were screened and finally 8 best primers were used for analysis.

Data analysis

The fingerprints generated by different primers were compared for their relatedness among isolates only bright bands were considered for scoring. The presence 1 and absence 0 of a band of particular molecular weight was scored as two alleles at a single locus to compile the binary matrics. The dendrogram was constructed by RAPDPLOT software.

RESULTS AND DISCUSSION

The total number of fragments amplified using eight RAPD primers is 89. The number of scorable markers

produced per primer ranged from 6 to 13 and size of the products ranged from 300 bp to 4 kb. Highest resolving power was shown by Primer OPN12 and least was shown by primer OPA10. Gene diversity ranged from 0.77 to 0.20. Primer OPN12 showed highest gene diversity and lowest gene diversity was shown by primer OPM6.

In case of ISSR total numbers of bands amplified were 85. Highest gene diversity was shown by primer UBC 820 and lowest by primers UBC 810. The size of amplified products ranged from 230 bp to 3500 bp. The number of scorable markers produced per primer ranged from 6 to 11. Gene diversity ranged from 0.7 to 1.3. Resolving power ranged from 3.8 to 10.8.

Dendrogram were generated by combining data from RAPD and ISSR (Figure 1). Phylogenetic tree can be divided into two major clusters A and B. Cluster A contains only one isolate and cluster B contains the rest. Cluster B can be further subdivided into clusters C and D. C contains single isolate while cluster D contains 28 isolates. Cluster D can be further subdivided into F and E. E contains 8 isolates and F has 21 isolates. Cluster F is further subdivided into G and H. G contains only 1 isolate and H contains 20 isolates. H is further subdivided into J and I; I contains 9 isolates and J 11 isolates. Similarly distance matrix has been generated for different isolates using combined data of RAPD and ISSR. Similarity coefficient of different isolates ranged from 0.22 to 0.66. Genetic distance was computed for all isolates.

The amount of genetic variation found in *A. alternata* using RAPD and ISSR is relatively high. Morris *et al.*[16] analyzed tomato isolates using RAPD primers showing high level of genetic variability. Molecular work presented here and other molecular study[1,16] confirm that even isolates collected from single species, the level of variation among isolates is high. Natural chance mutations along with the fact that rate of spore production and parasexual cycles are responsible for high rate of mutations. There is no known sexual cycle in *A. mali* and it is not known if the parasexual cycle occurs regularly in the genus *Alternaria*[6]. But truly asexual fungi are extremely rare and most population showed some influence of recombination[19]. Analysis of RAPD and ISSR profile showed no significance difference of *A.mali* isolates according to geographically origin. Lack of clustering in dendrogram indicates that various geographical subpopulations are not genetically isolated. Dissemination of spores by biotic and abiotic may be the cause of even distribution of genetic variation[18]. Samples collected from different cultivars of apple were unable to produce different cultivar specific pattern. It indicates that there are no cultivar specific strains. This study is first to use RAPD and ISSR markers to assess genetic diversity of *A. alternata* pathotype from Kashmir.

Such a large variation indicates that pathogen can survive in adverse environmental conditions thus pathogen can counteract control measures such as fungicide. Future studies will be done using more reliable markers like AFLP, ITS etc. to understanding of the population structure.

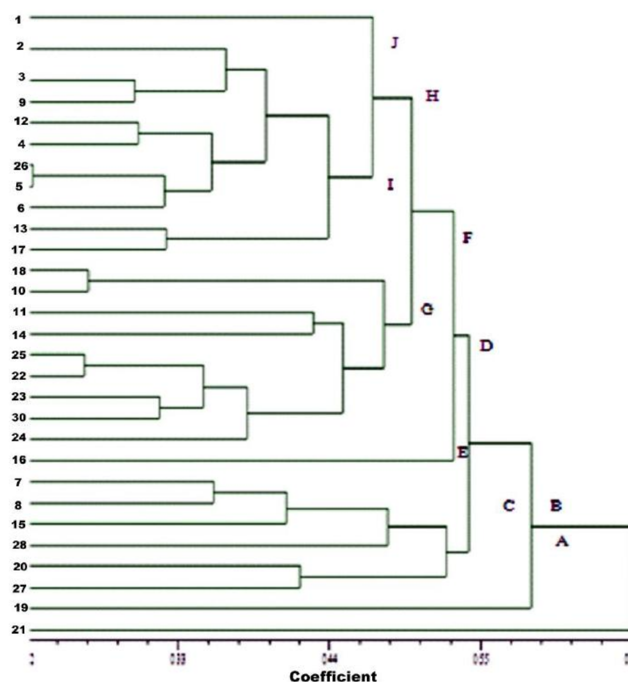


Figure 1 : Dendrogram generated using data of RAPD and ISSR

ACKNOWLEDGMENTS

Authors are grateful to the Department of Biotechnology (DBT), Govt. of India for funding the project on Apple. YPK is also thankful to Council of Scientific and Industrial Research (CSIR) for financial assistance in the form of research fellowships. Thanks are due to Coordinator, Bioinformatics Centre, University of Jammu for providing the necessary facilities.

REFERENCES

[1] Y.Adachi, H.Watanabe, K.Tanabe, N.Doke, S.Nishimura, T.Tsuge; Appl.Environ.Microbial., **59**, 3197-3205 **(1991)**.
[2] B.Andersen, E.Kroger, R.G.Roberts; Mycol.Res., **105**, 291-299 **(2001)**.
[3] D.Appel, T.R.Ordon; Mol.Plant Microbe Intract., **9**, 125-138 **(1996)**.
[4] M.Asari, A.Takahashi; Bulletin of the akita fruit tree experiments station, Japan, **19**, 13-24 **(1988)**.
[5] A.Bulajic, N.Filajdic, M.Babovic, T.B.Sutton; Plant Disease, **80**, 709 **(1996)**.
[6] D.E.L.Cooke, J.W.Foster, P.D.Jenkins, D.G.Jones, D.M.Lewis; Ann.Appl.Biol., **132**, 197-209 **(1998)**.
[7] J.J.Doyle, J.L.Doyle; Focus, **12**, 13-15 **(1990)**.
[8] D.F.Farr, G.F.Bills, G.P.Chamuris, A.Y.Ossman; American phytopathogical society, st. Paul, USA **(1989)**.
[9] N.Filajdic, T.Sutton; Phytopatholog, **82**, 1279-1283 **(1992b)**.
[10] N.Filajdic, T.Sutton; Plant Dis., **76**, 126-130 **(1992a)**.
[11] N.Filajdic, T.Sutton; Plant Dis., **75**, 1045-1048 **(1991)**.
[12] R.Hatta, K.Ito, Y.Hosaki, T.Tanaka, A.Tanaka, M.Yamamoto, K.Akimitsu, T.Tsuge; Genet., **161**, 59-70 **(2002)**.
[13] R.D.Johanson, L.Johanson, K.Kohmoto, H.Otani, C.R.Lane, M.Kodama; Phytopathology, **90**, 972-976 **(2000)**.
[14] L.Johnson, R.D.Johnson, H.Akamatsu, A.Salamiah, H.Otani, K.Kohmoto, M.Kodama; Curr.Genet, **40**, 65–72 **(2001)**.
[15] C.H.Kim, W.D.Cho, S.C.Kim; Korean Journal of Plant Protection, **25**, 221-228 **(1986)**.
[16] K.A.H.Morris, S.Mackerness, T.Page, C.F.John, A.M.Murphy, J.P.Carr, V.Buchanan-Wollaston; Plant J., **23**, 677-685 **(2000)**.
[17] K.Sawamura; *Alternaria* blotch. In: A.L.Jones, H.S.Aldwinckle (Eds); Compedium of apple and pear diseases. St Paul, MN, USA: APS Press, 24-25 **(1990)**.
[18] L.W.Tiffany, R.H.David, J.C.Barbara; Mycologia, **90**, 813-821 **(1998)**.
[19] I.E.Taylor, K.D.Hyde, E.B.G.Jones; New Phytologist, **142**, 335-346 **(1999)**.