

Pathogenic and molecular characterization of *Fusarium moniliforme* Sheld, the incitant of Fusarium maize stalk rot in the Punjab State of India

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Abstract

Fifty six isolates of *Fusarium moniliforme* collected from different maize cultivars grown in various regions of Punjab were characterized for their pathogenic variation and molecular diversity. Based on the multivariate cluster analysis of pathogenicity data, seven clusters were formed, each representing a specific disease reaction to a particular maize hybrid/inbred line used in this study. The isolate Fm 10 was found the most virulent with an average disease index (ADI) of 66.6% whereas, Fm 45 was found least virulent with ADI of 42.5%. The sub-montaneous undulating region of Punjab represented maximum percentile of least virulent isolates, however, undulating plain region of Hoshiarpur and Shaheed Bhagat Singh Nagar district of the State represented the most virulent zone of *F. moniliforme* isolates. Genetic diversity in the Punjab populations of *F. moniliforme* was studied using twenty random amplified polymorphic DNA (RAPD) markers, out of which 18 showed amplification with a total of 220 amplified fragments. Primer OPT-12 was found to be highly polymorphic with PIC value of 0.913 while OPT-9 was least polymorphic. The size of amplified DNA fragments ranged from 0.1-2.0 kb. Dendrogram based on molecular data generated by 18 RAPD primers showed six clusters with similarity percentage ranging from 36 to 96% and one independent lineage with 26% similarity coefficient. No correlation was obtained between the genetic diversity and pathogenic variation; however the latter was influenced by agro-climatic zones of north-western India. This was the first attempt to study the genetic diversity of *F. moniliforme* causing Fusarium stalk rot of maize in Punjab.

Keywords: *Fusarium moniliforme*, maize, RAPD-PCR, stalk rot, virulence

Introduction

Maize (*Zea mays* L) is traditionally grown as summer and spring season crop in Punjab, north-western State of India. With the set up of CIMMYT Borlough Institute for South East Asia (CIMMYT-BISA) in this part of the world, the maize crop is going to get a boost in its production. However, the production is limited by a number of diseases, especially post flowering stalk rots (PFSR) are a major constraint to maize cultivation during both Kharif and spring season in Punjab. In India, eight fungi and three bacteria are reported to cause stalk rots (Raju and Lal, 1976), however in Punjab, PFSR complex is caused mainly by three fungi viz., *Fusarium moniliforme*, *Macrophomina phaseolina*, and *Cephalosporium maydis*, out of which *F. moniliforme* Sheld is of high economic importance (Khehra et al, 1982). Frequency of association of *F. moniliforme* with stalk rot infection is more as compared to *M. phaseolina* and *C. maydis* during both spring and Kharif seasons in Punjab (Kaur and Mohan, 2012).

F. moniliforme infects maize at post-flowering stage to maturity of the crop. The disease causes internal decay and discoloration of the stalk tissue, directly reducing the yield by blocking translocation

of water and nutrients, and can result in death and lodging of the plants (Zaidi and Singh, 2005). In addition, Nelson et al (1992) and Rheeder et al (2002) have shown that strains of *F. moniliforme* isolated from maize are known to produce fumonisins B1 and B2 which may pose serious health risks, if consumed with human food or animal feed.

Resulting yield losses may occur either directly due to light weight and poor filling of ears or indirectly at harvest because of stalk breakage and lodging. Christensen and Wilcoxon (1966) reported an average yield loss of 7.5 - 15.0% from stalk rot in USA, whereas, Lu et al (1995) observed that 10 to 60% infected plants caused yield loss up to 25% in China. In India, the estimated loss in grain yield may go upto 13.4% (Payak and Sharma, 1985). Apart from yield loss, stalk rot induces lodging that further reduces yield besides reducing grain quality (Ledencan et al, 2003).

The genus of *Fusarium* is well known for taxonomic difficulties in defining and identification of species within, at the morphological level. To overcome this, pathogenic and molecular characterization have proven to be more reliable in the assessment of genetic diversity within *Fusarium* spp, Bacon et al

(1994) reported that maize isolates of *F. moniliforme* consisted of a series of aggressive biotypes and the effect of each on a cultivar varied. The differential aggressiveness of *F. moniliforme* isolates demonstrated considerable variability in pathogenicity of the fungus to maize seedlings, which if subjected to additional cultivars, the isolates might indicate physiological specialization or races (Asran and Buchenauer, 2003). At molecular level, PCR based random amplified polymorphic markers (RAPD) are the most commonly used tool to define microorganisms at species and intraspecific levels (Williams et al, 1990).

Little information is available on the extent of diversity of *F. moniliforme* isolates from this important maize growing belt of India, although several workers (Al-Amodi 2006; Amoah et al, 1996) elsewhere have demonstrated morphological, pathological and molecular variability of *F. moniliforme* isolates. In the present study, we demonstrated pathogenic and genetic diversity among the Punjab populations of *F. moniliforme*. This information will be highly useful in enhancing FSR resistant maize breeding programme and in developing suitable management strategies in Punjab by the scientists either at BISA or Punjab Agricultural University, Ludhiana.

Materials and Methods

Fungal isolates, their isolation and maintenance

Fifty six isolates of *F. moniliforme*-infected maize plants were collected from farmers' fields in different maize growing regions of Punjab during Kharif (rainy) and spring season during 2009-10 (Supplementary Table 1). Morphological identification was carried out according to Nelson et al (1983) and Leslie and Summerell (2006). Further, the identity of *F. moniliforme*

was confirmed with the help of species specific primers (Murillo et al, 1998). Each isolate was maintained as pure culture on potato dextrose agar (PDA) using single spore isolations and stored at 4°C (Choi et al, 1999). Each isolate was maintained by periodical transfer to PDA slants.

Pathological characterization of isolates

Maize inbreds/hybrids

For studying the reaction type of all 56 isolates of *F. moniliforme* collected from Punjab, a set of both resistant and susceptible maize inbred lines (LM-13, CM-140, CM-143, CML-25 and CM-600) and hybrids (PMH 1, JH 3459, NK 6240 and 31-Y-45) were cultivated during both Kharif and spring season 2011. Seed of all these inbred lines and cultivars were sourced from Maize Section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, India. Seed were sown in lines with row to row and plant to plant spacing of 60 cm and 20 cm, respectively. The plot size consisted of three rows of 2 meter length for each isolate. The crop was managed as per recommended Package of Practices for Kharif and spring season of PAU, Ludhiana (Anonymous 2011).

Inoculations

The stock culture of each isolate was prepared on Czapek's Dox Broth (HiMedia Laboratories) using toothpick method (Jardine and Leslie, 1992). Round hard bamboo toothpicks of about 5.5 cm length were boiled in water for one hour to remove resin, gum or any other toxic substances inhibitory to fungal growth. After boiling, toothpicks were stacked loosely in culture bottles keeping the tapering ends upwards. The level of broth was so adjusted that it

Table 1 - PIC value of primers and number of fragments amplified in RAPD analysis of isolates of *Fusarium moniliforme*.

Primer	Primer sequence	Total nr of amplified fragments	Nr of polymorphic bands	Polymorphic information content (PIC) values	Unique band size/ Isolate
OPT-1	GGGCCACTCA	13	13	0.889	950bp/Fm11 250bp/Fm31
OPT-2	GGAGAGACTC	8	8	0.866	-
OPT-3	TCCACTCCTG	7	7	0.681	-
OPT-5	GGGTTTGGCA	13	13	0.901	-
OPT-6	CAAGGGCAGA	14	14	0.906	-
OPT-7	GGCAGGCTGT	13	13	0.890	1.5kbp/Fm49 100bp/Fm54
OPT-8	AACGGCGACA	14	14	0.907	-
OPT-9	CACCCCTGAG	6	6	0.669	-
OPT-11	TTCCCCGCGA	7	7	0.725	-
OPT-12	GGGTGTGTAG	16	16	0.913	-
OPT-13	AGGACTGCCA	10	10	0.836	-
OPT-14	AATGCCGCAG	14	14	0.853	-
OPT-15	GGATGCCACT	13	13	0.889	-
OPT-16	GGTGAACGCT	13	13	0.873	-
OPT-17	CCAACGTCGT	10	10	0.891	-
OPT-18	GATGCCAGAC	15	15	0.899	1.5kbp/Fm19
OPT-19	GTCCGTATGG	15	15	0.891	-
OPT-20	GACCAATGCC	19	19	0.908	650bp/Fm9
Total		220	220	0.855	-

covered one-third length of toothpicks. After autoclaving, the sterilized medium in culture bottles was inoculated with the representative isolate of *F. moniliforme* and incubated at $25 \pm 2^\circ\text{C}$ for seven days to get thick mycelia mat with spores/mycelium at the tips of these toothpicks. Ten plants in each maize line in three replications were inoculated at flowering stage with each of the isolates separately in the field by inserting a toothpick into 2 cm deep hole made in an oblique manner in the centre of second internode from ground level.

Disease measurement

At the time of maturity, lower internodes of inoculated maize plants were split open longitudinally to see the extent of pith damage in the form of shredding and pinkish discoloration. The infection was recorded on 1 - 9 rating scale as suggested by Payak and Sharma (1983):

- 1 - discoloration only at the point of inoculation/ discoloration just started;
- 2 - 25 to < 50% of the internode area discolored;
- 3 - 50 to < 75% of the internode area discolored;
- 4 - internode fully discolored;
- 5 - adjacent internode showing discoloration up to 50%;
- 6 - discoloration up to two internodes;
- 7 - discoloration up to three internodes;
- 8 - discoloration of four internodes;
- 9 - discoloration of up to five internodes and plant dries up.

The pathogenic variability of *F. moniliforme* was assessed on the basis of these reactions and the isolates were grouped into different clusters based on their virulence reaction spectrum.

Molecular characterization

DNA extraction

The mycelial mat from ten days old cultures of each isolate of *F. moniliforme* grown on Potato Dextrose Broth was dried and ground to a fine powder in liquid nitrogen by constant crushing using sterilized and chilled pestle and mortar. DNA from all 56 isolates of *F. moniliforme* was isolated using CTAB (cetyl trimethyl ammonium bromide) method as modified by Saghai Maroof et al (1984). Isolated DNA was purified by adding RNase (Promega Inc) to a final concentration of $10 \mu\text{g ml}^{-1}$. The quantity and quality of DNA was checked by using TECAN 2000 Nanoquant Plate reader. The DNA of all the samples was diluted to $25 \text{ ng}/\mu\text{l}$ in nuclease free water and stored at -20°C for further use.

PCR amplification

A set of twenty RAPD primers (Integrated DNA Technologies, Coralville, USA) was used in the present investigations for amplification of 56 isolates of *F. moniliforme* (Table 1). *In vitro* amplification using polymerase chain reaction (Saiki et al, 1988) was performed in a 96 well micro titer plate in an Eppendorf Master Cycler ProS. PCR amplification was car-

ried out in a final volume of $25 \mu\text{l}$, containing 2.5 mM MgCl_2 , 1 unit Taq DNA polymerase, 0.1 mM dNTPs, $1\times$ PCR Buffer, $1 \mu\text{M}$ Primer, and 50 ng of genomic DNA. PCR amplification of each reaction sample was performed in Eppendorf Master Cycler ProS with 35 cycles of following thermal profile: initial denaturation at 94°C for 4 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute, extension at 72°C for 2 minutes, and final elongation at 72°C for 3 minutes. The amplified products were electrophoresed in 1.5% agarose gels in $0.5\times$ TBE buffer (Tris base 45 mM , Boric acid 45 mM , and EDTA 1 mM), stained with ethidium bromide at a concentration of $0.5 \mu\text{g ml}^{-1}$ of buffer. PCR products were resolved by gel electrophoresis at 5 V cm^{-1} for 2 - 3 hours. The gels were visualized under UV light and photographed using SYNGENE gel documenta-

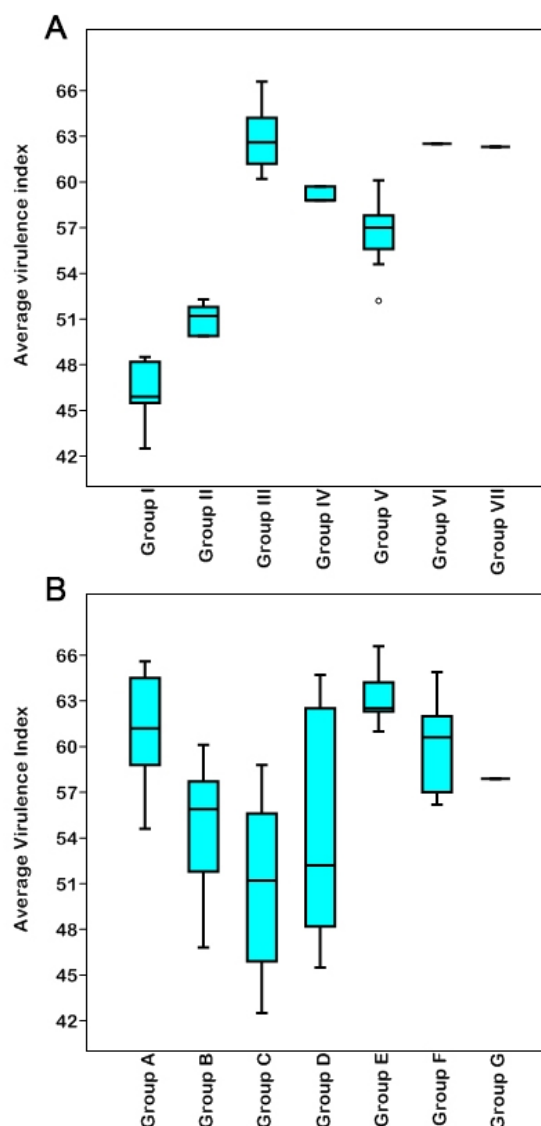


Figure 1 - A: Average virulence index of different clusters of *Fusarium moniliforme* maize isolates; B: their relationship with RAPD based clusters.

tion system with «Gene Snap» software programme.

Data Analysis

DNA fingerprint data generated by RAPD markers were converted into binary matrix form as 1 representing the presence and 0 the absence of band. The RAPD allele sizes were ascertained by comparing them with the known marker (100 bp ladder, Promega Inc). To measure the informativeness (discriminatory power), the polymorphic information content (PIC) was calculated for each RAPD marker by using the formula: $PIC = 1 - \sum (P_{ij})^2$, where P_{ij} is the frequency of j^{th} allele in i^{th} primer and summation extends over 'n' patterns (Botstein et al, 1980). Statistical analysis for RAPD data was conducted using Unweighted Pair Group Method based on Arithmetic Averages (UPGMA) in the software programme PAST (Paleontological statistics software package for education and data analysis) ver 3.02a (Hammer et al, 2001). Similarity matrices were calculated using Jaccard's coefficient.

Results

The results of pathological characterization of 56 isolates of *F. moniliforme* on both maize hybrids and inbred lines indicated different virulence spectra (Supplementary Table 1). The average virulence index (AVI) of different isolates was found lowest on maize hybrid PMH 1 (23.9 - 49.4%) and highest on Pioneer hybrid, 31-Y-45 (75.8 - 97.0%). In case of maize inbreds, mean disease severity was recorded least on LM-13 (21.6 to 52.2%), moderate on CM-143 (36.0 to 71.1%) and CML-25 (32.6 to 85.1%) and maximum on CM-140 (52.2 to 84.3%) and CM-600 (51.2 to 83.4%).

On the basis of AVI on different maize cultivars, isolates of *F. moniliforme* were divided into five clusters and two out groups (Fm 15 and Fm 11) in a dendrogram generated using UPGMA (Supplementary Table 2). Cluster-I was differentiated from cluster-II by its susceptible and moderately susceptible reaction to hybrids JH 3459 and NK 6240 respectively compared with highly susceptible reaction of cluster-II on these two hybrids (Supplementary Table 3). Except for cluster-I and II, all the other clusters showed highly susceptible reaction to inbreds CM-140 and CM-600. Cluster-IV and V differed from each other with respect to highly susceptible reaction given by NK 6240 to cluster-IV isolates and susceptible (52.8%) reaction to cluster-V isolates. The isolates grouped in clusters-III and Fm 15 isolate gave highly susceptible reaction to inbred CM-143 and these can be further differentiated with respect to moderately resistant reaction of Fm 15 on LM-13. Only one isolate (Fm 11) developed susceptible reaction (52.2%) on LM-13 whereas all other clusters produced moderately resistant to moderately susceptible reaction.

During the present investigations, cluster-III consisting of 22 isolates was found to be highly virulent, showing average virulence of 60.2 to 66.6% on

different tester lines (Figure 1A). The isolate Fm 10 was found to be the most virulent isolate with AVI of 66.6%. The isolates forming independent lineages, Fm 11 and Fm 15 were also observed to be highly pathogenic with mean AVI of 62.5 and 62.3 per cent, respectively. These three highly virulent isolates were collected from the Mahilpur block of district Hoshiarpur in the Punjab State. The isolates in cluster-II, IV and V were intermediate in their pathogenicity. However, the isolates grouped in cluster-I were weakly pathogenic with an AVI of 46.2%.

Response of individual pathogenic groups to different hybrids and inbreds of maize were depicted in Figure 2. Among maize hybrids and inbreds, frequency of *F. moniliforme* isolates exhibiting moderately resistant (MR) reaction was highest on LM-13 followed by PMH 1. Isolates showing maximum virulence frequency were observed on 31-Y-45 followed by two inbreds CM-600 and CM-140. On two maize hybrids NK 6240 and JH 3459, the isolates had different virulence spectra.

To investigate the degree of genetic diversity among different isolates of *F. moniliforme*, a total of 20 RAPD markers were used. DNA fingerprinting of 56 isolates of *F. moniliforme* collected from different maize growing areas of Punjab showed a dis-

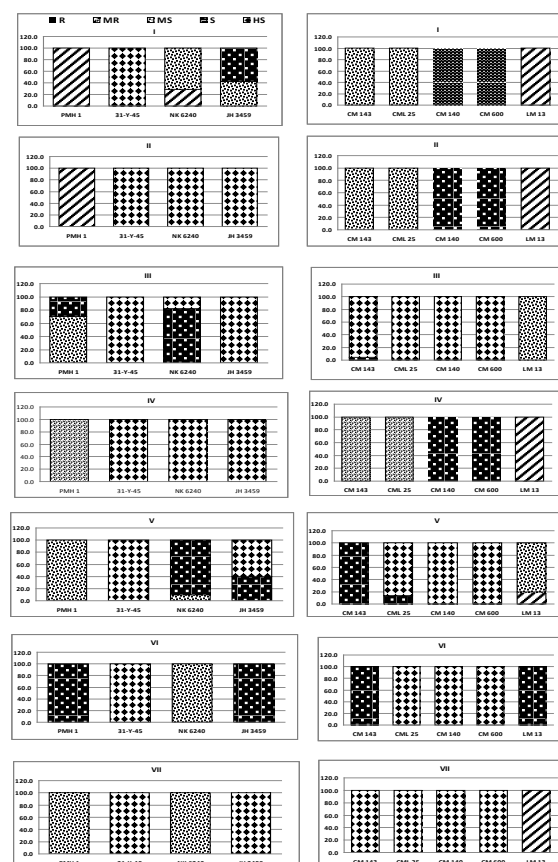


Figure 2 - Response of individual pathogenic groups to different maize inbreds and hybrids.

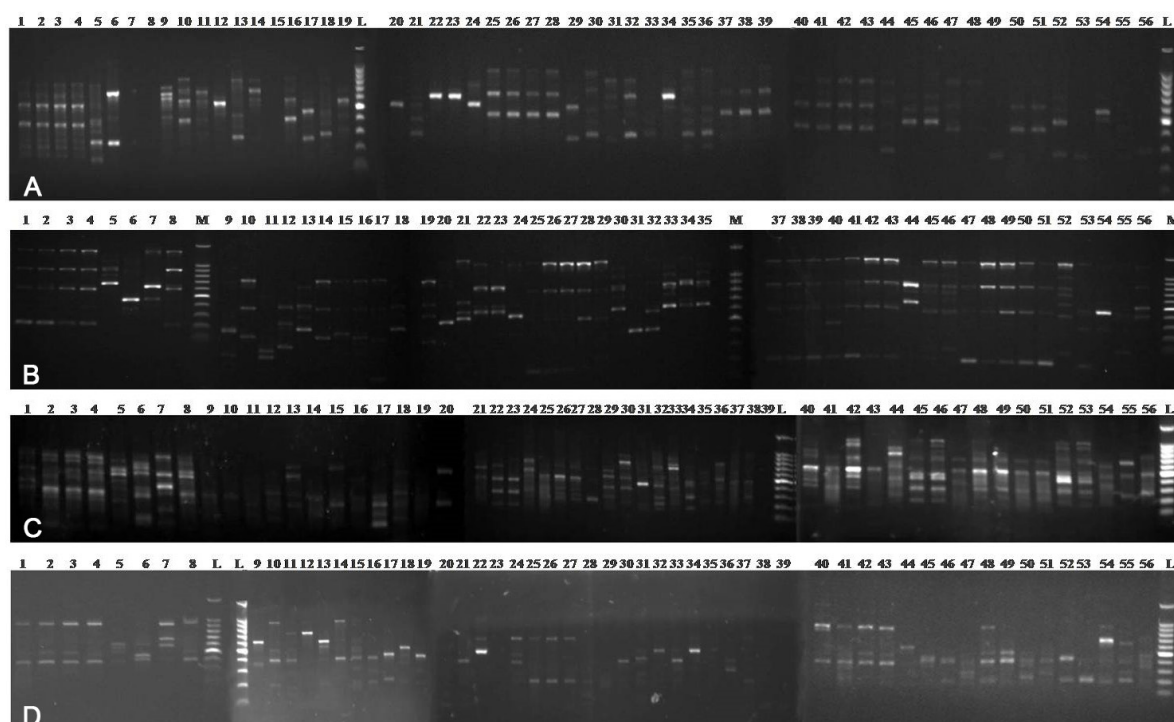


Figure 3 - PCR amplification products of *Fusarium moniliforme* isolates obtained by RAPD primers **A**: OPT-20; **B**: OPT-8; **C**: OPT-17; **D**: OPT-19.

tinct banding pattern. Out of 20 RAPD primers used, 18 showed amplification with total of 220 amplified fragments (Table 1). All the amplified fragments were polymorphic. The number of scorable bands obtained by using RAPD primers ranged from 6-19 on different primers. The primer OPT-20 amplified maximum (19) number of fragments. The fragments obtained were amplified within a range of 100 bp to 1.5 kb (Figure 3A). A specific band of 650 bp was obtained in isolate Fm 9 indicating its variability from rest of the isolates (Table 1). Two other primers viz., OPT-1 and OPT-7 can set apart Fm 11, Fm 31, Fm 49 and Fm 54 from other isolates as these produce specific amplification products unique to these isolates. Similarly, Fm 19 can be distinguished by OPT-18 as it produced a unique band of 1.5 kb size. The banding patterns obtained by primer OPT-8 in isolates Fm 5, Fm 6, Fm 9, Fm 54, and Fm 55 were completely different indicating their variability from rest of the isolates, whereas banding patterns obtained by isolates Fm 1, Fm 2, Fm 3 and those obtained by Fm 33, Fm 34, Fm 35 and Fm 37, Fm 38, Fm 39, Fm 41, Fm 42, and Fm 43 were identical indicating their greater similarity (Figure 3B). In case of primer OPT-17, except Fm 28, Fm 31 and Fm 39, all the isolates had many closely spaced bands in a ladder like pattern (Figure 3C). With primer OPT-19, fifteen bands of size ranging from 100 bp to 1.5 kb were amplified and all were polymorphic (Figure 3D). In the present investigation, PIC values ranged from 0.669 (OPT-9) to 0.913 (OPT-12). Hence, primer OPT-12 was most informative fol-

lowed by primers OPT-20, OPT-8, OPT-6 and OPT-5 whereas primers OPT-9 and OPT-3 were least informative.

Dendrogram constructed with UPGMA using the molecular data generated by 18 RAPD primers is presented in Figure 4. Grouping revealed one independent lineage formed by isolate Fm 55 with 15 per cent similarity coefficient. The rest 55 isolates were grouped into two major clusters; the first major cluster (39 isolates) was re-grouped into four small groups (A, B, C, and D) consisting of 9, 13, 5 and 12 isolates, respectively with genetic similarity value ranging from 24 - 90%, 32 - 94%, 24 - 91%, and 21 - 88%, respectively. The isolates Fm 50 and Fm 51 depicted the highest genetic similarity of 94%, followed by Fm 1 and Fm 2 with a genetic similarity value of 90%. The second major cluster (16 isolates) was separated into two sub-clusters (E and F) consisting of eight isolates each and had genetic similarity range of 21 to 70% and 21 to 76%, respectively.

Discussion

Ledencan et al (2003) and Szoke et al (2009) demonstrated the differences in the pathogenicity of *F. moniliforme* isolates on maize cultivars. Significant genotypic variation was observed in isolates of *F. verticillioides* from Philippines and all were found pathogenic but varied in the degree of aggressiveness (Cunha et al, 2011). The present studies had shown diversity within 56 *F. moniliforme* isolates collected from different maize growing areas of Punjab based

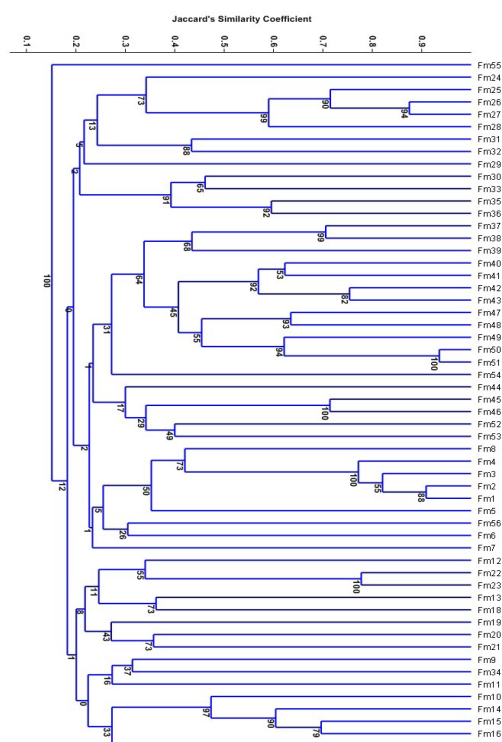


Figure 4 - Dendrogram showing molecular diversity among *Fusarium moniliforme* isolates using RAPD markers.

on pathogenicity and molecular markers.

Based on virulence spectra, the two isolates-Fm 11 and Fm 15 formed out groups owing to their unique pathological behaviour towards maize inbreds. Rest of the isolates were grouped in five clusters with cluster-III having highest AVI on all the cultivars suggesting existence of different pathological strains of the pathogen. Each cluster represents a specific disease reaction towards maize hybrids and inbred lines which forms the basis of differentiation into different groups.

The host pathogen interactions of *F. moniliforme* population studies indicated that highly susceptible reactions (40.9%) predominated followed by susceptible (29.3%) and moderately susceptible (23.0%) reactions. However, the isolates showing moderately resistant response accounted for only 6.8 per cent of the total isolates. High percentage of isolates having virulent alleles are known to change the genetic structure of pathogen population in such a way that even moderately resistant responses can progress towards susceptibility. And, the abundance of susceptible hosts in turn favors the more aggressive strains of the pathogen (Berngruber et al, 2013).

The present investigations have shown that virulence pattern of different isolates of *F. moniliforme* appeared to be influenced by agro-climatic regions of Punjab (Figure 5). About 81.2% of highly virulent isolates of *F. moniliforme* were localized in undulating plain region of Punjab State. However, about 77% of least virulent isolates were found in sub-mountain

undulating region and 61.5% of virulent isolates were found in Central plain zone of Punjab. Undulating plain region of District Hoshiarpur, Shaheed Bhagat Singh Nagar represented the most virulent zone of *F. moniliforme* isolates. This suggested that the variation in aggressiveness within population is influenced by the geographical distribution of isolates. On comparing the virulence frequencies of isolates on maize hybrids and inbreds with different agro-climatic regions, minimum frequency of virulent isolates were recorded on PMH 1 and LM 13 from sub-mountain undulating region and central plain region respectively (Figure 2). LM-13 being the female parent of PMH 1 hybrid is the contributing resistance source. In addition, temperature and humidity are the major contributing factors influencing the development of *Fusarium* fungi. Its incidence is often correlated to different climatic conditions in different geographic locations (Popovski and Celar, 2013). In contrast, Ittu and Ciocazanu (2011) reported that aggressiveness in *Gibberella fujikuroi* was found in all isolates of maize regardless of their geographic origin in South Romania.

RAPD markers have been successfully used to analyze isolates of *Fusarium* species collected from all over the world (Voigt et al, 1995). In the present study, RAPD analysis suggested that *F. moniliforme* isolates were highly variable genetically. Diverse gene pool in the genus with high genetic variability existed among the recovered *F. moniliforme* species. From the molecular analysis of *F. moniliforme* isolates, it is clear that the isolates from a particular geographical area tend to group together irrespective of their virulence pattern. The geographical isolation of each isolate contributed to genetic diversity revealed by molecular markers. The isolate Fm 55 was the only isolate collected from Gurdaspur district of Punjab State. The same was observed to be an out group in the molecular data generated. The isolates present in cluster III and V were from Hoshiarpur district which is an indicative of genetic similarity based on site specific grouping of the isolates. Kini et al (2002) clustered 28 isolates of *F. moniliforme* into 5 groups using cluster analysis with UPGMA and reported that isolates from the same host species were found to group together in the same cluster. Likewise, As-

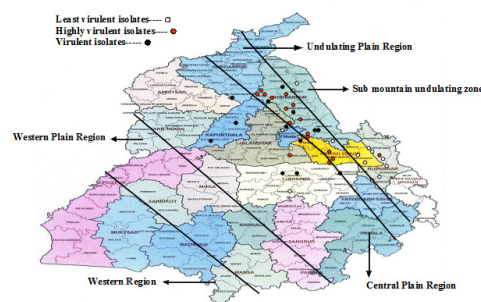


Figure 5 - Origin of *Fusarium moniliforme* isolates of maize from different agro-climatic zones of Punjab.

sigbetse et al (1994) reported a correlation between genetic similarity in a RAPD analysis of *F. oxysporum* and geographic origin. In contrast, Yli-Mattila et al (1996) found no correlation between isozymes and RAPD-PCR patterns and geographic origin or particular host plant infected.

Relationship between the pathotype and molecular marker based grouping was observed to be complex with different lineages representing one or more pathotypes/virulent phenotypes falling in more than one lineage. Perusal to the Figure 1A, it was observed that cluster III having maximum AVI (60.2 - 66.6) is represented in five genetic lineages. The highly virulent isolates Fm 11 and Fm 15 (from Hoshiarpur) which were grouped as independent lineages pathogenically were grouped together during genetic analysis in Figure 4. Based on pathogenicity, maximum variability was observed in S (susceptible) reaction types followed by HS (highly susceptible) types whereas reaction type MS (moderately susceptible) was observed only in cluster C. Cluster E and cluster B contained all the isolates showing HS and S reaction types, respectively (Figure 1B). Average disease index was least (50.8%) in the isolates contained in cluster C and maximum (63.0%) in isolates contained in cluster E. It could be concluded from the present pathotypic and genetic diversity data that origin of virulence in *F. moniliforme* is polyphyletic i.e., similar virulence can arise independently in distant geographical areas.

On comparing the clusters obtained by the data of pathogenic and molecular variability, no association could be obtained among virulence pattern and RAPD pattern. However, pathogenic and genetic variation of isolates is influenced by agro-climatic zones of North western India and the conclusions are in accord with those obtained by Abdel-Sattar et al (2003) and Satyaprasad et al (2000). This distribution of populations among different geographical zones will be useful in developing resistant breeding programme as well as providing more insights into molecular evolution of virulence genes in the pathogen.

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