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Full Research Article

Morphological, Cultural, Pathological and Molecular Variability among *Fusarium oxysporum* f.sp. *zingiberi* isolates

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Abstract

Nineteen isolates of *Fusarium oxysporum* f.sp. *zingiberi* causal organism of Fusarium yellows in ginger were collected from different ginger growing areas of Himachal Pradesh and designated as I_1 to I_{19}. Morphological variations with respect to mycelial colour, conidial size and formation of chlamydospores and pathogenic variation in terms of disease incidence among different isolates was studied in these isolates. The mycelial colour varied from white to dull white with slightly pinkish tinge. The microconidial size varied from 5.20×4.00 µm (I_8) to 12.30×5.70 µm (I_3) whereas macroconidial size varied from 16.20×4.70 µm (I_7) to 32.0×5.70 µm (I_4). Chlamydospore dimensions also varied in all the nineteen isolates of the test pathogen. All isolates formed chlamydospores on PDA medium. Pathogenic variability revealed that lesion size varied from 8.50 to 18.00 mm after 10 days of inoculation whereas, incubation period varied from 11 to 19 days. Genetic variation was also analysed by using forty 10 – mer oligonucleotide RAPD primers, out of which 2 primers yielded informative, strong and reproducible DNA amplicons of *Fusarium oxysporum* f.sp. *zingiberi* and thus were selected for studying the variation among isolates. The dendrogram of DNA fingerprints revealed 0 to 80% variation among isolates. All isolates were grouped into two different major groups each comprising of ten and nine isolates, respectively.

1. Introduction

Ginger is one of the most important cash crops of Himachal Pradesh. It is used as a flavouring agent, preservative in soft drinks. This crop is grown by small and marginal farmers of north eastern regions of India, Himachal Pradesh, Karnataka, Kerala, Orissa as well as South East Asian countries, Africa and Hawaii (Kumar et al*.,* 2008)., *Fusarium* yellows caused by *Fusarium oxysporum* f.sp. *zingiberi* is the most serious problem among various diseases affecting ginger. It has been found in most ginger growing areas of Himachal Pradesh. Plants infected by the fungus do not wilt rapidly but are stunted and yellowed. The lower leaves dry out over an extended period of time. The rhizomes show a creamy brown discolouration of the water-conducting vessels of the rhizomes and a prominent black dry rot of the tissues of the cortex. This is a dry rot characterized by collapse of the cortical tissues, occasionally accompanied by a purpling of the infected areas of the rhizome and a white cottony mycelial growth on the cut surfaces of ginger pieces.

Fusarium species are reputed for their complexity and capacity for rapid change. Even the successive single spore sub-cultures of wilt types have been reported to exhibit variations in growth type, pigmentation and pathogenicity (Awuah and Lorbeen, 1988). Variation in morphological, cultural and pathogenic characteristics among isolates of some *forma specialis* in *Fusarium* is well established (Booth, 1971). The morphological, cultural and biochemical parameters traditionally used to study the variability in plant pathogens are influenced by host age, inoculum quality and environmental conditions. Moreover, these techniques are time consuming and laborious. The molecular markers, on the other hand, are highly efficient and accurate for studying genetic variability in plant pathogens (Sharma et al., 1999). Keeping this in view, the present studies were carried out to investigate the molecular variability among the *Fusarium oxysporum* f.sp. *zingiberi* isolates using RAPD markers. In addition, the variation

in cultural and morphological characteristics, pathogenic variability of *Fusarium oxysporum* f.sp. *zingiberi* isolates on ginger was also investigated.

2. Materials and Methods

2.1. Cultural and morphological characteristics

Nineteen isolates of *Fusarium oxysporum* f.sp. *zingiberi* were collected from different parts of Himachal Pradesh and were designated as I_1 to I_{19}. In order to study the cultural and morphological characteristics, mycelial bits (5 mm diameter) of each isolate were taken from the actively growing cultures and centrally placed on Petri plates (90 mm) containing sterilized PDA medium. Plates were incubated at 25±1ºC for 2 weeks. Data in relation to cultural characteristics and average microconidial, macroconidial and chlamydospores size were recorded.

2.2. Pathogenic variability

For ascertaining the pathogenic variability of *Fusarium oxysporum* f.sp. *zingiberi* isolates, selected healthy rhizomes were surface sterilized with 0.1% mercuric chloride solution and washed in distilled water. Each rhizome was inoculated with uniform sized cultural bits in the centre. These rhizomes were kept in Petri plates with moist blotting paper, incubated at 25±1^{o}C. Each treatment was replicated thrice. The length of resultant brown lesions was measured after 10 days.

2.3. RAPD analysis of pathogen isolates

Mycelium of all 19 isolates was grown in potato dextrose broth at 25±1^{o} C for 14 days and was harvested by filtration through Whatman No. 1 filter paper. For DNA isolation 250 mg lypholized mycelium of each isolate was ground in liquid nitrogen in an Eppendorf tube (2.0 ml) and total fungal genomic DNA was isolated according to CTAB (Hexa-decyl tri-methyl ammonium bromide, Sigma Chemical CO., St. Louis, USA) method (Murray and Thompson, 1980). Isolated DNA was purified by using 5µl RNase A (10mg ml^{-1}) and quantified by running 2µl of each DNA sample on 1.0% agarose gel to get desired concentration. Initially, forty 10-mer random primers of A and D series of Operon Technologies were evaluated (Table 3) for amplification of two isolates, and two primers (OPAD 14 and OPAD 15) giving maximum polymorphs were selected for further studies of all the isolates.

The PCR reaction was set up with a total reaction volume of 25 µl comprised of 2µl (20 ng) fungal genomic DNA, 2.5 µl PCR buffer (10 X) containing $MgCl_2$ (15 mM), 1 µl of 10 mM dNTP, 0.2 µl of Taq DNA polymerase (3 U μl^{-1}), and 2 µl (15 ng) each of 10-mer oligonucleotide primer (Operon Technologies). The reactions were performed in a thermocycler (Applied Biosystems 9700) set for initial denaturation at 94°C for 2 min, 45 cycles of 94°C at 1 min, 37°C for 1 min and 72°C for 2 min and final extension at 72°C for 10 min. PCR amplification products were electrophoretically separated on 1% agarose gel stained with EtBr in TAE buffer and photographed under UV light in a Gel Documentation System (Alpha Infotech Corporation).

Isolates were screened twice with primers OPAD 14 and OPAD 15. Repeatable polymorphisms were scored as 1 for presence of a band and 0 for absence. 1kb DNA ladder was loaded in two lanes on agarose gel for determining the band size. The resulting matrix was used to compute DICE's similarity coefficient for all pairwise comparisons of the isolates (Dice, 1945). Cluster analysis by the unweighted pair-group method of arithmetic averages (Sneath and Sokal, 1973) were performed with the similarity values using the SAHN procedure of the program NTSYS-pc (Rohlf, 1993).

3. Results and Discussion

3.1. Cultural and morphological characteristics

The mycelial colour varied from white to dull white with slightly yellowish (I_2) to pinkish tinge (Table 1). The microconidial size varied from 5.20×4.00 µm (I_8) to 12.30×5.70 µm (I_3) whereas macroconidial size varied from 16.20×4.70 µm (I_7) to 32.0×5.7 µm (I_4). Chlamydospore dimensions also varied in all the nineteen isolates of the test pathogen. All isolates formed chlamydospores on PDA medium. Chlamydospores dimensions varied from 6.50×5.12 (I_2) to 10.88×7.90 (I_{15}). Variation in cultural and morphological characteristics of *Fusarium oxysporum* f.sp. *zingiberi* isolates has earlier also been reported by Dohroo and Sharma (1992) and Siddiqui and Kaushal (2000).

3.2. Pathogenic variability

Studies on pathogenic variability revealed that lesion size varied from 8.50 to 18.00% (Table 2). Maximum lesion size was observed in I_5 (18.00 mm) followed by I_{11} (16.00 mm) and I_4 (16.00 mm), whereas, minimum lesion size was observed in I_7 after 10 days of inoculation. Incubation period varied from 11 (I_1, I_8, I_{12}, $I_{15,}$ I_{17}) to 19 (I_7, $I_{11,}$) days. Out of nineteen isolates, six isolates (I_2, I_8, I_{14} I_{15}, I_{16}, I_{17}, and I_{19}) exhibited incubation period of 14 days representing one group in terms of pathogenesis. Pathogenic variation in isolates of *F. oxypsorum* f. sp. *zingiberi* was observed by various workers (Rana and Arya, 1991; Dohroo and Sharma, 1992; Siddiqui and Kaushal, 2000). Bauxton (1956) suggested that *Fusarium oxysporum* has a system resembling the parasexual cycle of other fungi imperfecti, which allow different nuclei occasionally to exchange genetic material, may be one factor responsible for the development of new races.

3.3. Molecular variability

Screening of forty 10-mer oligonucleotide RAPD primers

Table 1: Variation in cultural and morphological characteristics of *Fusarium oxysporum* f.sp. *zingiberi* isolates

Isolate	Cultural characters	Morphological average size (µm)		
		A	B	C
I_1 (Nauni)	Mycelium white, dense, uniform, pinkish to violet tinge in the colony	9.2×4.5	30.7×4.6	8.2×7.3
I_2 (Pandah)	Mycelium white, fluffy, light yellow tinge in the colony	9.8×4.7	28.2×4.2	6.5×5.1
I_3 (Dharja)	Mycelium white dense, fluffy, colony raised and slightly pinkish at the centre	12.3×5.7	27.0×4.3	8.4×6.6
I_4 (Nohra)	Mycelium white, dense, uniform, pinkish tinge in the colony	10.2×4.3	32.0×5.7	8.6x 7.3
I_5 (Matyana)	Mycelium white, uniform raised and dense	8.7×4.8	24.0×4.3	6.6×6.1
I_6 (Charna)	Mycelium dense, fluffy, dull white, formation of violet ring at the periphery and in the centre of the colony	9.7×4.6	21.8×4.5	9.0×7.1
I_7 (Ghanduri)	Mycelium dull white, dense, uniformly raised at the centre with pinkish tinge	9.6×4.2	16.2×4.7	8.3×7.3
I_8 (Bhangri)	Mycelium white, uniform, dense, appearance of violet colour at the centre, suppressed growth at the periphery	5.2×4.0	20.9×5.0	8.4×7.8
I_9 (Gaura)	Mycelium white, uniform, raised, dense, slightly pinkish tinge at the periphery of the colony	9.8×4.7	26.5×3.9	6.8×5.0
I_{10} (Chayavan)	Mycelium dull white, uniform, fluffy, light pinkish tinge in colony	7.2×3.1	27.8×4.8	7.9×5.0
I_{11} (Kahan)	Mycelium white, fluffy, uniform and dense	7.9 × 4.2	24.9×4.6	8.8×7.8
I_{12} (Birla)	Mycelium white, uniform, fluffy and sparse	5.2 × 4.3	22.5×4.2	7.6×7.1
I_{13} (Nehli)	Mycelium white, uniform and fluffy	8.2 × 3.1	29.2×4.8	8.0×7.1
I_{14} (Dheera)	Mycelium white, uniform, dense, formation of violet colour, suppressed growth at the periphery	7.9 × 4.5	24.8×4.5	8.0×6.6
I_{15} (Kanda)	Mycelium white, dense uniformly raised	7.2×3.1	19.3×3.8	10.8×7.9
I_{16} (Shamoga)	Mycelium white, uniform, dense, formation of violet colour, suppressed growth at the periphery	7.9×4.2	27.5×5.2	9.6×7.7
I_{17} (Didag)	Mycelium white, dense, uniform and fluffy	5.2×4.3	23.1×4.9	9.6×7.7
I_{18} (Malgaon)	Mycelium white, fluffy, uniform and dense	8.2×3.1	22.3×5.6	9.8×9.1
I_{19} (Khadri)	Mycelium white, uniform in centre and suppressed at the periphery	7.9×4.5	24.0×5.3	7.7×7.0

A: Microconidia; B: Macroconidia; C: Chlamydospores

revealed all primers to yield strong and reproducible DNA bands of *F. oxysporum* f sp *zingiberi* isolates by PCR (Table 3). The level of polymorphism varied with different primers in two isolates. Maximum number of bands (14) was scored with primer OPAD 15 followed by 12 bands with OPAD14. DNA fingerprints obtained with RAPD primer OPAD 15 is shown in Figure 1. Multivariate analysis was conducted to generate a similarity matrix using DICE coefficient (Table 4). A dendrogram was constructed with 228 fragments (including 65 bands of different isolates) obtained from RAPD primer OPAD 15 as shown in Figure 2 by UPGMA analysis to estimate genetic diversity and relatedness among nineteen isolates of *F. oxysporum* f.sp. *zingiberi*. The maximum coefficient (100.0) was observed for the groups of isolates I_1 and I_2 as well as I_{13} and I_{14} (Table 4). The dendrogram (Figure 2) produced from computerized cluster analysis of the DNA fingerprints

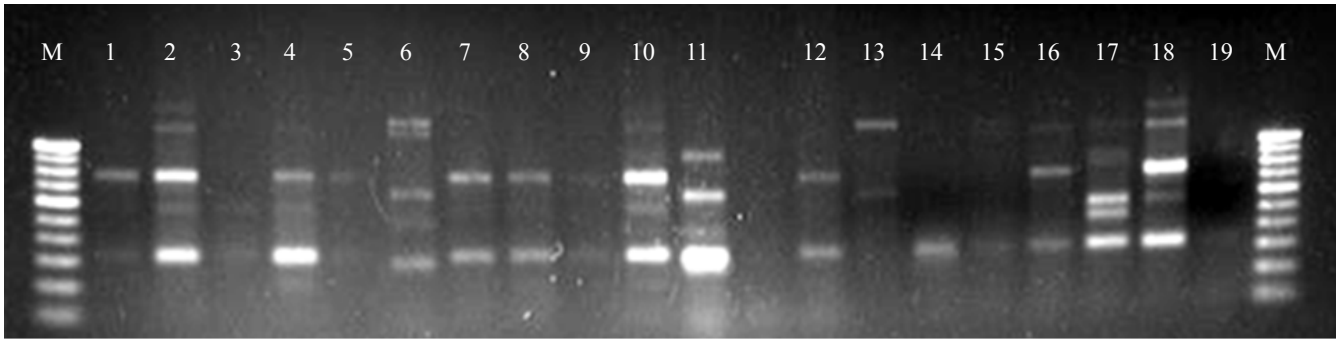

Figure 1: Amplification products of *Fusarium oxysporum* f.sp. *zingiberi* isolates with primer OPAD-15. Lane M : 1kb ladder (molecular weight marker); Lanes 1 to 19: Isolate No 1 to 19,.

Table 2: Pathogenic variability of *Fusarium oxysporum* f.sp. *zingiberi* isolates on ginger rhizomes

Isolate	Lesion size (mm)	Incubation period (days)
I_1 (Nauni)	10.0	11
I_2 (Pandah)	12.0	14
I_3 (Dharja)	14.0	15
I_4 (Nohra)	16.0	14
I_5 (Matyana)	18.0	13
I_6 (Charna)	9.0	14
I_7 (Ghanduri)	8.5	19
I_8 (Bhangri)	10.0	11
I_9 (Gaura)	14.0	14
I_{10} (Chayavan)	15.0	17
I_{11} (Kahan)	16.0	19
I_{12} (Birla)	12.0	11
I_{13} (Nehli)	15.0	14
I_{14} (Dheera)	14.5	12
I_{15} (Kanda)	14.0	11
I_{16} (Shamoga)	12.0	14
I_{17} (Didag)	14.0	11
I_{18} (Malgaon)	9.5	17
I_{19} (Khadri)	13.0	15

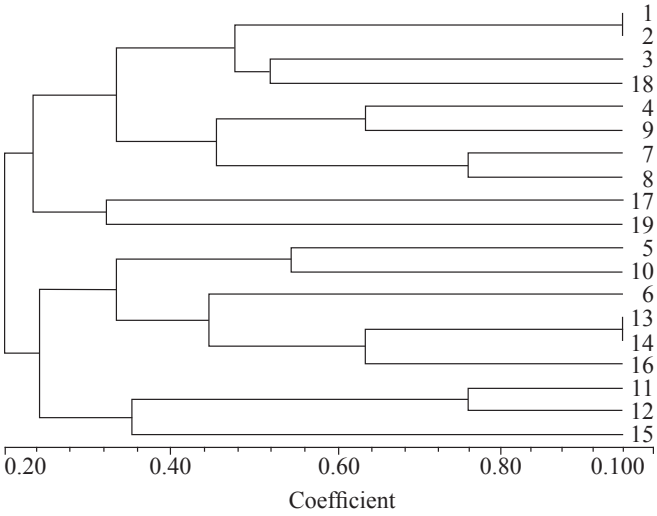

Figure 2: Dendrogram derived from banding patterns of RAPD analysis with primer OPAD 15

revealed a great deal of heterogeneity among the isolates. All the isolates were classified into two major groups which separated from each other at 20% similarity coefficient value. Group I comprised of isolates 1, 2,3,18,4,9,7,8,17 and 19 while, Group II comprised of isolates 5,10,6,13,14,16,11,12 and 15.

Differential hosts, cultural characteristics, morphological differences and biochemical tests are the traditional markers used to study the variability in plant pathogens. These markers distinguish pathogens on the basis of their physiological characters i. e. pathogenicity and growth behaviour. However, these markers are influenced by the age of the host, inoculum quality and environmental conditions. However, RAPD

Table 3: Primer, its sequence and amplification detected during preliminary studies on primer selection.

Sl. No.	Primer	Sequence (5' to 3')	Amplification	Number of amplicons
1	OPAA 1	CAGGCCCTTC	Yes	7
2	OPAA 2	TGCCGAGCTG	Yes	6
3	OPAA 3	AGTCAGCCAC	Yes	7
4	OPAA 4	AATCGGGCTG	Yes	8
5	OPAA 5	AGGGGTCTTG	Yes	5
6	OPAA 6	GGTCCCTGAC	Yes	8
7	OPAA 7	GAAACGGGTG	Yes	6
8	OPAA 8	GTGACGTAGG	Yes	5
9	OPAA 9	GGGTAACGCC	Yes	7
10	OPAA 10	GTGATCGCAG	Yes	5
11	OPAA 11	CAATCGCCGT	Yes	4
12	OPAA 12	TCGGCGATAG	Yes	5
13	OPAA 13	CAGCACCCAC	Yes	3
14	OPAA 14	TCTGTGCTGG	Yes	5
15	OPAA 15	TTCCGAACCC	Yes	3
16	OPAA 16	AGCCAGCGAA	Yes	6
17	OPAA 17	GACCGCTTGT	Yes	4
18	OPAA 18	AGGTGACCGT	Yes	5
19	OPAA 19	CAAACGTCGG	Yes	7
20	OPAA 20	GTTGCGATCC	Yes	6
21	OPAD 1	ACCGCGAAGG	Yes	7
22	OPAD 2	GGACCCAACC	Yes	5
23	OPAD 3	GTCGCCGTCA	Yes	6
24	OPAD 4	TCTGGTGAGG	Yes	4
25	OPAD 5	TGAGCGGACA	Yes	7
26	OPAD 6	ACCTGAACGG	Yes	4
27	OPAD 7	TTGGCACGGG	Yes	4
28	OPAD 8	GTGTGCCCCA	Yes	3
29	OPAD 9	CTCTGGAGAC	Yes	5
30	OPAD 10	GGTCTACACC	Yes	6
31	OPAD 11	AGCGCCATTG	Yes	8
32	OPAD 12	CACCGTATCC	Yes	6
33	OPAD 13	GGGGTGACGA	Yes	8
34	OPAD 14	CTTCCCCAAG	Yes	12
35	OPAD 15	CATCCGTGCT	Yes	14
36	OPAD 16	AGGGCGTAAG	Yes	8
37	OPAD 17	TTTCCCACGG	Yes	5
38	OPAD 18	GAGAGCCAAC	Yes	6
39	OPAD 19	CTGGGGACTT	Yes	8
40	OPAD 20	ACCCGGTCAC	Yes	8

Table 4: Similarity matrix for dice coefficient of *Fusarium oxysporum* f.sp. *zingiberi* isolates.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1.0000																		
2	1.0000	1.0000																	
3	0.5000	0.5000	1.0000																
4	0.3333	0.3333	0.3000	1.0000															
5	0.3333	0.3333	0.2142	0.1111	1.0000														
6	0.2857	0.2857	0.1666	0.4000	0.2222	1.0000													
7	0.2857	0.2857	0.4000	0.4000	0.1000	0.3333	1.0000												
8	0.2500	0.2500	0.5000	0.6000	0.0909	0.2857	0.8000	1.0000											
9	0.4000	0.4000	0.2000	0.6666	0.1250	0.5000	0.5000	0.4000	1.0000										
10	0.2857	0.2857	0.1666	0.1666	0.5714	0.3333	0.1428	0.1250	0.2000	1.000									
11	0.1250	0.1250	0.1666	0.1666	0.2222	0.3333	0.3333	0.2857	0.2000	0.3333	1.0000								
12	0.1111	0.1111	0.1538	0.1428	0.2000	0.2857	0.2857	0.2500	0.1666	0.2857	0.8000	1.0000							
13	0.1666	0.1666	0.0909	0.2500	0.2857	0.5000	0.2000	0.1666	0.3333	0.5000	0.2000	0.1666	1.0000						
14	0.1666	0.1666	0.0909	0.2500	0.2857	0.5000	0.2000	0.1666	0.3333	0.5000	0.2000	0.1666	1.0000	1.0000					
15	0.3333	0.3333	0.1818	0.2000	0.2500	0.4000	0.1666	0.1428	0.2500	0.4000	0.4000	0.3333	0.2500	1.0000	1.0000				
16	0.3333	0.3333	0.1818	0.2000	0.2500	0.4000	0.1666	0.1428	0.2500	0.4000	0.1666	0.1428	0.6666	0.6666	0.2000	1.0000			
17	0.2857	0.2857	0.2727	0.1666	0.2222	0.3333	0.3333	0.2857	0.2000	0.3333	0.0000	0.0000	0.2000	0.2000	0.1666	0.1666	1.0000		
18	0.5000	0.5000	0.5454	0.2500	0.2727	0.2222	0.5714	0.5000	0.2857	0.3750	0.3750	0.3333	0.1250	0.1250	0.2500	0.2500	0.3750	1.0000	
19	0.1250	0.1250	0.1666	0.1666	0.0000	0.3333	0.3333	0.2857	0.2000	0.0000	0.1428	0.2857	0.0000	0.0000	0.1666	0.0000	0.3333	0.2222	1.0000

analysis has been successfully used to identify strains and races in phytopathogenic fungi (Logrieco et al., 1990; Chiocchetti et al., 1999). It has been used for studying inter and intraspecific variability among population from different and same geographic areas (Walker et al., 2001). DNA amplification fingerprinting analysis of genetic variation within *Fusarium oxysporum* f.sp. *zingiberi* and *Fusarium oxysporum* f.sp. *pisi* has also been reported by Pappalardo et al. (2009) and Gupta et al. (2011), respectively.

4. Conclusion

The RAPD pattern visualizes variations in total DNA and thus is suitable for differentiating *F. oxysporum* f.sp. *zingiberi* isolates. Results obtained from RAPD analysis in present study enabled quicker and precise variability analysis for *F. oxysporum* f.sp. *zingiberi* isolates. Understanding genetic structure of *F. oxysporum* f.sp. *zingiberi* pathogen populations through present studies may provide deeper insight into epidemiology and evolutionary potential of this pathogen and could lead to development of improved strategies for managing Fusarium Yellows disease in ginger.

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