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Wheat Seed-Borne Mycoflora, Pathogenicity of *Fusarium moniliforme* Isolates and their Molecular Characterization

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Abstract: *Nine samples of wheat grains cv. Sakha 69, were screened for the associated fungi, 15 fungal species belonging to eight genera were isolated from wheat seeds. The isolated fungi were Aspergillus flavus, Aspergillus niger, Aspergillus ochraceous, Aspergillus parasiticus, Alternaria alternata, Stemphylium sp., Cladosporium sp., Drechslera sp. Fusarium solani, Fusarium moniliforme, Fusarium semitectum, Fusarium nivale, Fusarium oxysporum, Penicillium spp., Trichoderma sp. The genus Aspergillus gave the highest percentage of seed colonization of the isolated fungi followed by Fusarium spp. Pathogenicity test for nine isolates of F. moniliforme was conducted under greenhouse conditions to investigate the levels of infection against wheat cv. Sakah 69 and the percentage of infection was used as a criteria to evaluate the pathogenicity of F. moniliforme isolates against wheat seedlings. Cluster analysis was a reliable method to differentiate between nine isolates belonging to the genus Fusarium. Three different primers were used in the following study to differentiate among F. moniliforme isolates and the third primer proved to be the best in grouping F. moniliforme isolates into numerous distinct groups.*

Key Words: *Wheat (Triticum aestivum L.), seed-borne mycoflora, RAPD technique, pathogenicity of F. moniliforme.*

I. Introduction

Numerous injuries and stresses, affect on wheat plants at all stages of growth, which interfere with their normal functioning and development. Each year wheat losses are increased due to seed infection and contamination [1] and [2].Therefore a seed health technique usually plays an important role for successful cultivation and yield exploitation of a crop species. From various factors that affect seed health, the most important are the seed borne fungi that lower seed germination and reduce seed vigor resulting in low yield and quality [3]. Earlier investigations reported that seed plays an important role not only for successful cultivation but also for increasing yield of crop. Numerous seed-borne pathogens of grain crops are responsible to cause variation in plant morphology and also reducing yield [4] and [5]. *Fusarium* sp. is one of the most important genera of plant pathogenic fungi, with a record of devastating infections in many different kinds of economically important plants [6]. Numerous crops all over the world are attacked by *Fusarium* spp. which are responsible for wilt blights, root rots and cankers**.** In recent years the use of various nucleic acid-based techniques for the detection and identification of microorganisms has dramatically increased and may provide high specific tools for molecular studies [7] and [8]. Restriction fragment length polymorphisms (RFLPS), which are costly and time consuming, can be used; however, the random amplified polymorphic DNA (RAPD) is also a suitable technique to evaluate taxonomic identity. The application of (RAPD) to produce isolate-specific DNA-finger prints is especially promising [8] and [9]. This technique has the advantage of that no DNA base sequence information of the organism is needed. The technical simplicity and speed of RAPD methodology is a principal advantage over other techniques [7].

The aim of the present study was to survey wheat seed-borne fungi, test the pathogenicity of some isolates against wheat cv. Sakha 69 and to compare the molecular characterization for these isolates by using RAPD technique.

II. Materials and Methods

Source of seed samples and isolation

Nine seed samples with a bad disease history of wheat cv. Sakah 69 were obtained from different locations in Egypt. A random subsample of 100 wheat seeds was surface sterilized in 2.5% Clorox solution for 2 minutes, and washed several times in sterilized water [10] and [11]. Twenty five sterilized wheat seeds of cv. Sakha 69 were randomly selected and placed on 8.5cm Petri dishes on PDA medium, each was replicated 4 times; plates were incubated for 12-hr darkness at 20±2^{o}C for 7 days. After incubation each colony was examined macroscopically or microscopically for identification the genus or species level according to [12], [13] and [14]. Isolation frequency and percentage of infection was recorded and tabulated according [11].

Pathogenicity test of nine *F. moniliforme* isolates against wheat grains

Substrates for growth of each *F. moniliforme* isolate; previously isolated from wheat grains were prepared in 500ml glass bottles; each bottle contained 50g of sorghum grains and 40 ml of tap water. The bottles were autoclaved for 30 minutes. Isolate inocula, were taken from one-week-old culture on PDA were aseptically introduced into the bottles and allowed to colonize sorghum grains for 3 weeks. Batches of autoclaved clay loam soil were inoculated separately with inoculums of each isolate at the rate of 50g/kg of soil. Infested soil was dispended in 25cm-diameter clay pots and these were planted with 20 seeds per pot for apparently healthy; and the surface sterilized wheat grains cv. Sakha 69 to insure that the source of infection were only from the artificial inoculation with *Fusarium moniliforme* isolates. For control treatment sterilized sorghum grains were mixed thoroughly with soil at a rate of 50g/kg of soil. Pots were randomly distributed on a greenhouse bench under a temperature regime ranged from 20±2^{o}C to 24±2^{o}C. Percentage of infection was recorded after 45 days of planting [15].

Molecular Characterization

Extraction of genomic DNA of nine *F. moniliforme* isolates

DNA was extracted from 50mg of mycelium from nine *F. moniliforme* isolates using Qiagen kit for DNA extraction. The extracted DNA was dissolved in 100 µl of elution buffer .The concentration and purity of the obtained DNA was determined by using "Gen qunta" system-Pharmacia Bio-tech. The purity of the DNA for all samples was 90-70% based on the 1.6 and 1.8 ratio. The DNA concentration was adjusted at 6 ng/µl for all samples in TE buffer pH 8.0.

Random amplified polymorphic DNA (RAPD) technique

RAPD amplification was carried out using three different random primers shown in Table (1) . Each 25µl reaction mixture contained 1 unit of *Taq* DNA polymerase, 0.2 mM each dNTP,1xPCR buffer,3mM $MgCl_2$, 10 pmol of primer and approximately 50 ng of template genomic DNA. PCR conditions was as follows: initial denaturation at 95^{o}C for 5 min, followed by 45 cycles of denaturation at 95^{o}C for 1 min, annealing at 36^{o}C for 1 min and extension at 72^{o}C for 2 min followed by a final extension cycle at 72^{o}C for 10 min then held at 4^{o}C. The amplified products were separated on 2% agarose gel at 75 constant volt, using 1x TBE buffer followed by staining with ethidium bromide solution (1µg/ml) and the bands were visualized with UV transilluminator. All gels were scanned for band R_f using gel documentation system (AAB).The M.W. of bands were determined against DNA marker Ameresco (100bp)-k800 and phenograms were based on un weighted pair-group method of arithmetic means (UPGMA).

Table (1): List of primers used for RAPD analysis in this study along with their sequences

Primer Code :	Sequences (5′--------- 3′)
Primer -1	CGTGCGGGAA
Primer -2	GTTT CGCTCC
Primer -3	GTAGACCCGT

Statistical analysis

The experimental design of pathogenicity test was a randomized complete block with replicates. Analysis of variance (ANOVA) of the data was performed with the Mstat-C package (A Micro-computer Program for the Design, Management, and Analysis of Agronomic Research Experiments, Michigan State Univ.,USA). Least significant difference (LSD) was used to compare means.

Gel analysis of the data

DNA gels were scanned for band R_f using gel documentation system (AAB Advanced American Biotechnology 1166). The different M.W. of band was determined against PCR marker Ameresco (100bp)-k800 by unweighted pair-group method based on arithmetic mean (UPGMA).

III. Results and Discussion

A total of 15 fungal species belonging to eight fungal genera were isolated Table (2) from wheat grains cv. Sakha 69 and the most frequent fungus were *Aspergillus* sp., followed by *Fusarium* spp., whereas the lowest fungus was *Trichoderma* sp. also, isolation yielded different species of fungi. Sample no.4 followed by sample no.6 yielded the highest percentage of fungi, while the sample no.3 and 8 yielded the lowest percentage of fungi. The other wheat samples yielded different percentages of fungi ranged from 15.1 to 18.5%, respectively. The occurrence of pathogen species are of central importance in the ecology of host-pathogen interactions in a complex pathosystems, *i.e*., those with multiple pathogens in a single or multiple hosts. Within such pathosystems, biotic and abiotic factors influence the distribution and abundance of such pathogen species [16],

[17] and [18]. Subsequently, patterns of association results from interrelationships among organisms and from environmental factors. Such these patterns depends on whether or not organisms selected or avoid the same habitat or have no interaction [14], [19], [20] and [21]. Therefore, pathogen species (e.g. seed-borne pathogens) were in a competition for a single resource (*e.g.*, a seed) tends to occupy the same niche. Such niche overlap generates affinity (or lack affinity) for coexistence among species, known as inter specific associations [3].

Table (2): Isolation and frequency (%) of fungi isolated from nine samples of wheat seeds cv. Sakha 69 on PDA medium.

Fungi	[a] S-1 %	S-2 %	S-3 %	S-4 %	S-5 %	S-6 %	S-7 %	S-8 %	S-9 %
Aspergillus flavus	16.9	13.9	15.9	14.1	12.2	15.7	7.0	22.6	9.5
Apergillus niger	7.8	7.7	25.0	11.9	10.8	14.6	10.5	24.2	5.9
Aspergillus ochraceus	9.1	15.4	0.0	9.8	4.0	13.5	11.6	21.0	13.1
Aspergillus parasiticus	0.0	7.7	6.8	1.1	6.7	11.2	15.1	8.1	9.5
Alternaria alternata.	0.0	0.0	4.5	2.2	8.1	8.9	7.0	0.0	7.1
Stemphylium sp.	12.9	4.6	2.3	3.3	0.0	6.7	8.1	0.0	3.6
Cladosporium sp.	14.3	4.6	0.0	6.5	9.5	0.0	4.6	0.0	8.3
Drechslera sp.	15.6	16.9	9.0	5.4	0.0	7.8	1.2	0.0	7.1
Fusarium solani	1.3	1.5	9.0	7.6	14.9	3.4	5.8	3.2	1.2
Fusarium moniliforme	5.2	18.5	13.6	9.8	5.4	14.6	7.0	6.4	10.7
Fusarium semitectum	1.3	0.0	0.0	15.2	4.0	2.2	0.0	1.6	0.0
Fusarium nivale	3.9	4.6	6.8	0.0	0.0	1.1	11.6	11.3	0.0
Fusarium oxysporum	0.0	0.0	4.5	8.7	6.7	0.0	3.5	0.0	11.9
Penicillium spp.	6.5	4.6	2.3	3.3	5.4	0.0	2.3	1.6	2.4
Trichoderma sp.	5.2	0.0	0.0	1.1	12.2	0.0	4.6	0.0	9.5

[a] S = Sample Number

Data shown in Table (3) indicate that the fungus *Aspergillus flavus* show the highest percentage of isolation frequency (14.2%), followed by *Apergillus niger* and *Apergillus ochraceus* at 13.2 and 10.8%, respectively. Whereas, the lowest percentage of isolation was noticed with the fungus *Fusarium semitectum* and *Penicillium* spp. at 2.7 and 3.2%, respectively.

Table (3): Frequency (%) of fungi isolated from nine Samples of wheat seeds cv. Sakha 69 on PDA medium.

Fungus	Mean Frequency (%)
Aspergillus flavus	14.2 [a]
Apergillus niger	13.2
Aspergillus ochraceus	10.8
Aspergillus parasiticus	7.4
Alternaria alternata.	4.2
Stemphylium sp.	4.6
Cladosporium sp.	5.3
Drechslera sp.	7.0
Fusarium solani	5.3
Fusarium moniliforme	10.2
Fusarium semitectum	2.7
Fusarium nivale	4.4
Fusarium oxysporum	3.9
Penicillium spp.	3.2
Trichoderma sp.	3.6

[a] Data recorded as mean (%) of nine samples of wheat seeds cv. Sakha 69 on PDA medium for each isolated fungus.

Data presented in Table (4) show the effect of 9 *F. moniliforme*, isolates on the percentage of pre-emergence and post-emergence damping-off of wheat seedlings, the investigated isolates show different significant levels of infections on wheat compared with control treatment. Isolate no.1 of *F. moniliforme* from Sharkiya governorate showed the least infection percentage at both pre-emergence and post-emergence stages (7.5 and15%, respectively) and the highest survival plants (77.5%).Whereas, isolate no.5 from Dagahiliya showed highest percentage of infection at both pre-emergence and post-emergence damping-off stages of wheat at 32 and 42.5%, respectively and the least survival plants at 25.5%. The similar trend was recorded with isolate no.7 of Damietta, pre-emergence and post-emergence damping-off was 32 and 43.5%, respectively and survivals (24.5%). Regarding the other parameters of plant growth in relative to infection, there are a noticeable

significant differences recorded in this respect *i.e.*, isolate no.1 and 8 from Sharkiya and Dagahiliya were the highest in plant height (cm) and dry weight (mg/plant) and yielded 17.37 and 18.28 cm/plant height and gave 92.5 and 97.25 (mg/plant) of dry weight, respectively. The obtained results are somewhat similar to those reported by [4] and [5]. In their scientific work it was reported that the association and isolation frequencies of some fungal species isolated from wheat grains were significantly correlated with seedling variables and the significance of some variables may indicate the presence of causal relationship between wheat seed-borne fungi and the incidence of seedling diseases.

Table (4): Effect of nine *F. moniliforme* isolates on wheat cv. Sakha 69 in artificially infested soil under greenhouse conditions

Isolate No.	Geographic origin	Pre-emergence damping-off %	Post-emergence damping-off %	Survival (%)	Plant height (cm)	Dry weight mg/plants)
1	Sharkiya	7.5	15	77.5	17.37	92.5
2	Gharbiya	27.5	40	32.5	13.37	77.53
3	Kafr El-sheikh	25	22	53	15.89	64.75
4	Behira	20	25	55	15.43	67.75
5	Dagahiliya	32	42.5	25.5	14.83	78.42
6	Behira	15	27	58	15.57	69.75
7	Damietta	32	43.5	24.5	13.45	74.00
8	Dagahiliya	10	20	70	18.28	97.25
9	Qualyubiya	30	39.5	30.5	14.82	80.25
10	Check	5	0.0	95.0	20.42	119.5
L.S.D at ($P \leq 0.05$)		9.95	15.0	13.42	0.83	10.68
L.S.D at ($P \leq 0.01$)		13.44	20.26	18.12	1.12	14.43

In the present study, wheat grains were planted in artificially infested soil with the investigated nine isolates of *F. moniliforme* under greenhouse conditions. Therefore, it seems reasonable to mention that the source of infection to wheat grains were only the *F. moniliforme* isolates which are the causal organism of seedling damping-off. The disease pressure during pre-emergence stage was higher than that during post-emergence stage for all the tested *F. moniliforme* isolates. In addition; pre-mergence damping-off was positively correlated with infection (Table 5). These results imply that the role of seed-borne fungi of wheat as seedling disease incidents was more evident in the pre-emergence stage compared with the post-emergence stage. The occurrence and associations of *F. moniliforme* are of great importance in the ecology of host-pathogen interactions within such pathosystems, biotic and abiotic factors influence the distribution and abundance of pathogen species. Subsequently, patterns of association results from interrelationships among organisms and from environmental factors. These patterns depends on whether or not organisms select or avoid the same habitat, have mutual attraction or repulsion, or have no interaction and these results are in agreement with that results reported by [22] and [23].

Table (5): Correlation [a] among variables used in evaluating pathogencity of nine isolates of *F. moniliforme* from wheat grains.

Variables	2	3	4	5
1- Pre-emergence damping–off (%)	0.895** [a]	0.969**	-0.835**	-0.515
2-Post-emergence damping–off (%)		-0.977 **	-0.844**	-0.274
3-Survival (%)			0.863**	0.396
4-Plant height (cm)				0.608
5-Dry Weight (mg/plant)				-

[a] Linear correlation coefficient (r) is significant at $p < 0.05$ (*) or $p < 0.01$ (**)

Phenogram in Fig.(1), using the average linkage cluster analysis between groups of nine *F. moniliforme* isolates relative to their pathogenicity on wheat cv. Sakha 69 show that the *F. moniliforme*, isolates were divided into two major groups, the first were four isolates of F5,F7,F2 and F9 and the second group were five isolates of *F. moniliforme* (1,8,4,6 and 3).

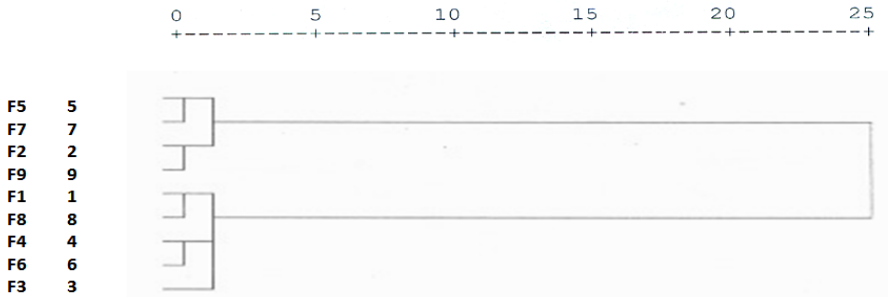

Fig (1): Phenogram using the average linkage cluster analysis between groups of nine *Fusarium moniliforme* isolates based on their pathogenicity (%) on wheat seedlings.

For studying the molecular variations among the investigated *F. moniliforme* isolates; three different 10 mer random primers were used. The molecular variations among nine isolates in the present study were varied. The RAPD profile of amplification products generated with primers; shown in Fig. (2) primer-1 revealed a degree of similarities among the 9 tested *F. moniliforme* isolates. Primer-1 revealed that Fusarium isolates can be grouped into a number of sub clusters. Isolates no 2 and 9 in similarity up to 10%, were grouped in one cluster and isolates no.3, 4 and 5 were grouped in another one cluster with similarity 15%, and the last cluster grouped was isolates no.6, 8 and 7 in similarity10%, whereas, isolate no.1 of Sharkiya were separated alone without any correlation with other isolates.

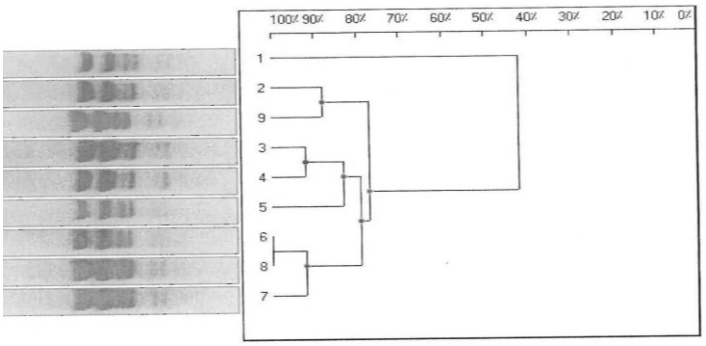

Fig. (2): Dendogram by UPGMA cluster analysis for DNA extracted from nine *F. moniliforme* isolates using RAPD (primer-1) .

The genetic variations among the nine isolates of *F. moniliforme,* by the RAPD profile of amplification products generated with primer-2; showed in Fig (3) primer-2 revealed a degree of similarities among the 9 tested *F. moniliforme* isolates. Primer-2 revealed that Fusarium isolates can be grouped into a number of sub clusters. Isolate no 1 and 3 are grouped in one cluster in similarity up to 10% and isolates no 2, 6 and 9 were grouped in another cluster with similarity 15%. Isolates no. 7, 8, 4 and 5 were grouped in one another sub-cluster of similarity up to 40%.

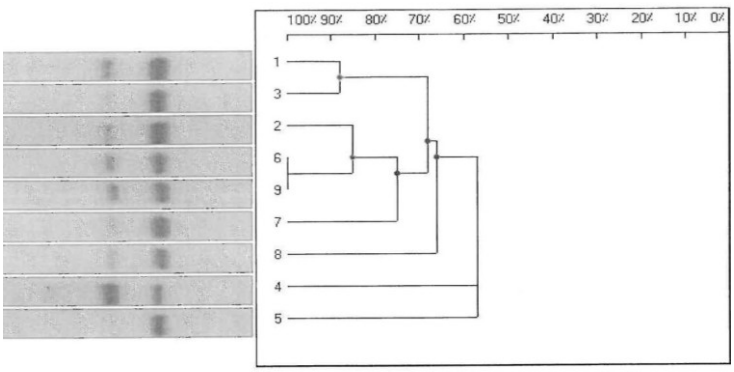

Fig. (3): Dendogram by UPGMA cluster analysis for DNA extracted from nine *F. moniliforme* isolates using RAPD (primer 2).

The genetic variations among the nine isolates of *F. moniliforme*, by the RAPD profile of amplification products generated with primer-3 showed in Fig (4) primer-3 revealed a degree of similarities among the 9 tested *F. moniliforme* isolates it can concluded that isolates can be grouped into a number of sub clusters.

Isolates no. 1 and 8 are grouped in one cluster and isolate no. 5 and 7 were grouped in another cluster in similarity 70%. Isolates no. 3 and 4 were grouped in another cluster in similarity up to 85%, and isolates no. 2 and 9 were grouped in a single cluster in similarity 85%.The dendogram in Fig.(4) show a high efficacy in grouping the investigated isolates and considered the best primer in this study.

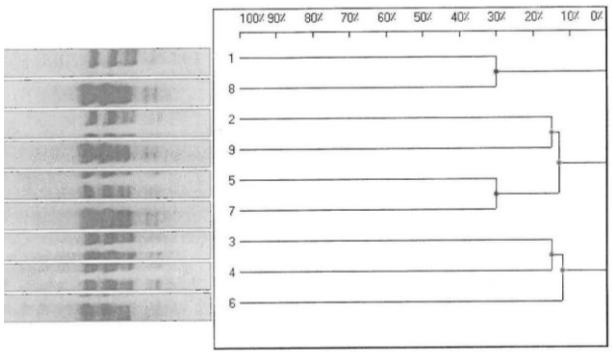

Fig. (4): Dendogram by UPGMA cluster analysis for DNA extracted from nine *F. moniliforme* isolates using RAPD (primer -3).

In the present study we have used RAPD-PCR technique to study the molecular diversity among nine *F. moniliforme* isolates relative to their pathogenicity and geographical distribution. DNA pattern from the isolates show that, there are a noticeable correlation among some isolates of *F. moniliforme* and the percentage of pathogenicity and grouped in one cluster *i.e.* isolate no.1 and 8 although they were from different governorates (Sharkiya and Dagahiliya). The primer-3 is different than the other primers in grouping the isolates and revealed the best grouping for the isolates *i.e.* isolates no.1 & 8 grouped in one cluster and they were similar in their pathogenicity. Isolates no.5 & 7 are grouped in one cluster and they were similar in their pathogenicity. This study provides a new insight to the concept on host-pathogen interactions. Also, gives an overview on the relationship between the pathogenicity of some important *F. moniliforme* isolates and the molecular diversity. [24] and [25]. Efficacy of primer-3 in grouping the tested isolates may be due to its ability to conjugate with different fragments of the extracted fungal DNA from nine *F. moniliforme* isolates. Differences in the pathogenicity of the investigated isolates in the present study depends on their virulence and the genetic structures, therefore a noticeable differences was shown in the percentages of infection against wheat seedlings; correlated sometimes with fungal phylogenetic characterization and these results were somewhat similar to that reported by [8] and [25].

IV. Conclusion

Among various seed-borne mycoflora that affect seed health, the most important are the *Fusarium* spp. that not only affect seed germination, but also reduce seed vigor resulting in low yield. Thus, healthy seed plays an important role not only for successful cultivation but also for increasing yield of wheat crop**.** These pathogens may affect the plant resulting in a reduction of the grain quality. Random amplified polymorphic DNA (RAPD) is also a suitable technique to evaluate taxonomic identity. The application of (RAPD) to produce isolate-specific DNA-fingerprints is especially promising. This technique has the advantage of that no DNA base sequence information of the organism is needed. The technical simplicity and speed of RAPD methodology is a principal advantage over other techniques. Therefore, further studies must be directed towards this kind of work.

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