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DETECTION OF FUMONISIN PRODUCING STRAINS OF *Fusarium moniliforme* CAUSING RICE BAKANAE DISEASE USING MOLECULAR MARKERS

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ABSTRACT

Fusarium spp. produce fumonisin, fumonisin consider one of the mycotoxin groups that contaminate feed and food products and posses maximum threat to human and animal health. Twelve *Fusarium* isolates were isolated from four Egyptian governorates, i.e, Kafrelsheikh, Dakahlia, Gharbia and Behera. One isolate (*F. verticillioides*) from infected corn, 10 isolates from infected rice with bakanae disease (*F. moniliforme*) and one *F. solani* isolate from infected wheat. Two specific primers named VERTF^{-1} and FUM1 were used in this study to PCR assay to discriminate fumonisin producing and non producing strains. The Inter Generic Spacer region (IGS) of rDNA coding units and polyketide synthase (PKS) gene FUM1. Eleven isolates were positive for VERTF^{-1} primer and proved to be potential fumonisin producers and one isolate (*Fusarium solani*) was scored negative for both primers. The primer FUM1 showed negative signal in five strains, one of *F. verticillioides* and five of *Fusarium moniliforme*. This present study provide quite rapid and specific method helps in accurate discrimination of fumonisin producing strains.

INTRODUCTION

Fumonisins are mycotoxins produced by both rice and maize pathogen *Fusarium moniliforme* (Karthikeyan *et al.*, 2011). Contamination of rice and rice products with fumonisins was threated agriculture and food safety world wide. Even though there are some reports pointed out that contamination of grains with fumonisins was associated with human esophageal cancer (Nelson *et al.*, 1993) in addition to neural tube defects, leukoence phalomalacia and edema as found in some populations from human, horses and swine, respectively (Hendricks, 1999; Marasas *et al.*, 2004).

Morphological characters of fumonisin, produced by fungal species, are not enough in some cases (Jurado *et al.*, 2010). DNA based molecular techniques were used to develop accurate and sensitive methods for identification isolates that can produce fumonisins. For example, polymerase chain reaction (PCR) technique was one of these techniques that can detect and identify *Fusarium* species which produce these toxins.

The intergenic spacer region (IGS) is a non-coding with high variable sequence (Moretti *et al.*, 2004) two set of primers (VERTF1/2) were detected

by (Patino *et al.,* 2004). In a previous research experiments, a PCR assay has been developed for fumonisin detection by FUM1 primer.

MATERIALS AND METHODS

Isolation of the causal organism:

The experiments of this work carried out during three successive growing seasons of 2013, 2014 and 2015 at the farm of Rice Research and Training Center (RRTC), Sakha, Kafr El-Sheikh, Egypt in the laboratory of plant pathology in addition to the lab of genetics Dept. Faculty of Agriculture, Tanta University. According to Hansen (1926), the diseased specimens were cut into 1 to 2 cm pieces followed by sterilization for two min with immersed in 0.5 % sodium hypochlorite solution (NaOH)and washed twice in sterilized dsH2O and then placed in plates contain potato dextrose agar medium (PDA).. Furthermore, The PDA plates were put in incubators for 5 to 7 days at 27±2 °C to let the fungi to grow. The growing fungi were purified by using hyphal tip or single spore techniques.

Identification of fungal isolates:

Morphological characteristics and microscopic examination were used to identify the selected isolates from fungus at the plant pathology laboratory in Rice Research & Training Center (RRTC), Sakha, Kafrelsheikh, Egypt through the key of imperfect fungi (Barnett and Hunter, 1972; Nelson *et al.,* 1983 and Summerell *et al.,* 2003).The isolates shown in Table (1) were supplied by Dr. W. Gabr (RRTC) .

Pathogenicity test:

The tested samples from rice materials were collected from different rice cultivars infected with bakanae disease symptoms from the previously mentioned governorates. Ten isolates of *Fusarium moniliforme* were isolated from infected rice cultivars, one isolate from corn (*Fusarium verticillioides*) and one from wheat (*Fusarium solani*) were used. Sakha 101 rice cultivar consider one of the most sensitive cultivar for rice bakanae disease, this cultivar grains were soaked in spore suspension of the tested isolates for two days (at con. 4 x 10^5 spores/ml) followed by incubation for another two days. Moreover, 50 grains from infected grains were seeded in plastic pots (15 x 15 cm. diameter), the seeded pots were arranged in rows and in three replicates in case of each isolate. The pots were placed in the greenhouse at 30-35°C and were fertilized with urea (46.5% N) 3g/pot one time. The control part included 50 grains soaked in 100 ml dsH2O fror two days followed by two days from incubation. The percentage of germination (%) for each infected plants with Bakanae disease and died plants (bakanae disease severity) were recorded at 30 days after sowing in against to before sowing. *Fusarium* isolates, location, identification and plant source are shown in Table (1).

Table (1). Fusarium isolates, location, identification and plant source

Isolate no.	Location (Governorate)	Morphological identification	Source of plant isolate	Cultivar
1	Kafrelsheikh	*Fusarium verticillioides*	Corn	-
2	Kafrelsheikh	*Fusarium moniliforme*	Rice	Sakha 101
3	Kafrelsheikh	*Fusarium moniliforme*	Rice	Giza 177
4	Kafrelsheikh	*Fusarium moniliforme*	Rice	Sakha 101
5	Dakahlia	*Fusarium moniliforme*	Rice	Sakha 101
6	Dakahlia	*Fusarium moniliforme*	Rice	Giza 177
7	Gharbia	*Fusarium moniliforme*	Rice	Sakha 101
8	Gharbia	*Fusarium moniliforme*	Rice	Giza 177
9	Kafrelsheikh	*Fusarium solani*	Wheat	Gemmiza 9
10	Beheria	*Fusarium moniliforme*	Rice	Sakha 101
11	Beheria	*Fusarium moniliforme*	Rice	Giza 177
12	Kafrelsheikh	*Fusarium moniliforme*	Rice	Giza 178

Fungal isolates and primers:

Isolates of *Fusarium moniliforme* were obtained from infected rice seed and stem samples form four different rice locations for rice fields in Egypt, during 2014 season Fusarium spp. Two primers of intergenic spacer region (IGS) were used according Baird *et al.*, 2008. Primer name and nucleotide sequence are located in Table (2).

Table (2): Primer name and nucleotide sequence

Primer name	Primer sequence	
VERTF-1/ VERTF-2	F5' GCGGGAATTCAAAAGTGGCC 3'	R 5' GAGGGCGCGAAACGGATCGG 3'
FUM1	F5'CCATCACAGTGGGACAAGT 3'	R 5'CGTATCGTCAGCATGATGTAGC 3'

DNA isolation and PCR condition:

The total genomic DNA was extracted from hypha (100 mg of homogenized hypha using liquid nitrogen) of each *Fusarium* isolate of the studied 12 isolates using the easy extraction kit (EZ-10 Spin Column Genomic DNA Minipreps Kit, plant), Bio Basic Inc. Canada, followed by an RNase-A treatment according to the manufacturer's instructions. DNA was quantified on 0.8% agarose gel using a known concentrations of λ uncut genomic DNA as standard. DNA concentrations of was adjusted up to 50 ng/µl.

Amplification of DNA was done according to manufacture instructions (CinnaGen/ Iran). Briefly, each 25µl reaction contained 12 µl of 2x PCR Master mix , 50 ng/ µl from the genomic DNA (1 µl), 2 µl of each primer (10 pmol/ µl), (in addition to 10 µl sterile deionized water. The PCR reactions were performed in Thermal Cycler (LongGene - MG96G / China) according to the following protocol. The initial denaturation was for 4 min at 94 C°, followed by 30 cycles of 1 min at 94°C, 1 min annealing temp (°C depending on primer) and 1 min at 72°C, plus a final extension of 5 min at 72°C, and the

reactions were held at 4°C. The PCR products (about 15 µl from each reaction) were loaded in 2 % agarose gel supplemented with ethidium bromide against 100 bp DNA ladder (Larova GmbH- Germany) as DNA size marker at 70 volt and the visualized via UV and photographed with gel documentation.. Gel Analyzer software package version 2010a was used to measure the molecular size of the amplified DNA fragments separated on gels..

RESULTS AND DISCUSSION

Pathogenicity of all isolates (twelve isolates) was tested on the most susceptible cultivar for bakanae disease (Sakha 101). Disease incidence percentage was estimated by counting number of the infected plants per hundred plants.

Data presented in Table (3) indicated that isolate no. one *(F. verticillioides*) and isolate no.9 (F. solani) were non pathogenic to rice bakanae disease. For bakanae disease percentage and severity, the highest infection was obtained from isolates no. 2 and 4 was isolated from Sakha 101 from Kafrelsheikh governorate, while the lowest infection were obtained from isolates no. 5,6 and 12 were isolated from Giza 177 and Giza 178 from Dakahlia and Kafrelsheikh governorates ,respectively (Table 2). Similar results were obtained by Bagga and Vineet-Kumar (2000) who mentioned that the rice infection with bakanae disease will be vary depending on the tested cultivars. On the other hand, Krishnaveni *et al.* (2001) reported that *Fusarium moniliforme* disease incidence and severity were correlated with the yield significantly and negatively when the infected plants were screened and selected.

Table (3). Pathogenicity of the twelve fusarium isolates on the rice cultivar Sakha 101 under greenhouse condition

Isolate no.	Location (Governorate)	Morphological identification	Source of plant isolate	Germination (%)	Bakanae disease (%)	Bakanae disease severity (%)
1	Kafrelsheikh	*F. verticillioides*	Corn	78.33	0.0	0.0
2	Kafrelsheikh	*F. moniliforme*	Rice	86.67	65.33	18.67
3	Kafrelsheikh	*F. moniliforme*	Rice	82.33	62.67	16.33
4	Kafrelsheikh	*F. moniliforme*	Rice	89.33	71.33	21.33
5	Dakahlia	*F. moniliforme*	Rice	85.33	26.33	9.67
6	Dakahlia	*F. moniliforme*	Rice	83.67	49.67	12.67
7	Gharbia	*F. moniliforme*	Rice	81.33	61.33	18.33
8	Gharbia	*F. moniliforme*	Rice	87.67	59.67	15.67
9	Kafrelsheikh	*F. solani*	Wheat	56.33	0.0	0.0
10	Beheria	*F. moniliforme*	Rice	85.67	62.33	17.67
11	Beheria	*F. moniliforme*	Rice	84.33	51.67	15.33
12	Kafrelsheikh	*F .moniliforme*	Rice	87.67	49.33	13.67
13	Control	*F. verticillioides*	Rice	90.33	1.33	00.33
L.S.D. 5%				3.125	1.235	1.231

DNA studies:

All 12 Fusarium isolates were tested with the set primers VERTF^{-1} and VERTF^{-2} to analyze their ability to produce fumonisin. Eleven isolates (one of *F. verticillioides* as a control and 10 isolates of *F. moniliforme*) showed the expected 400-bp amplicons. As expected no amplification was observed in the negative control DNAs (*F. solani*) (Fig. 1).

In this study the use of this set of primers showed that detected the occurrence of one isolate of *Fusarium solani* did not show amplification and, therefore, should not be potential Fumonisin producer. The VERTF$^{-1/2}$ set of *Fusarium* isolates used for their potential to produce fumonisin the excepted 400 bp amplicon was detected only with the DNA from 11 of 12 *Fusarium* isolates.

Compared to IGS primer the Fum-5 gene detection is more accurate and reliable detection method. Among the 12 isolates, five selected strains showed positive signal amplification of Fum-5 gene of 400 bp size fragments (lane no. 1, 4, 6, 8, 12).

In Conclusion, primer Fum-5 consistently produced a product of 400 bp that was effective for identification of *F. moniliforme* associated with rice tissues. *F. moniliforme* was clearly the dominant species and would probably be the main source of the fumonisin production in cereal sample according as reported by (Kedera *et al.,* 1998; Grim *et al.,* 1998).

Previous workers (Baird *et al.,* 2008 and Srinivasa *et al.,* 2008) used this primer only with maize infecting *Fusarium* tissues. This study is the first effort on rice in Egypt represented banding size for both VERTF and Fum 5 primers.

Karthikeyan *et al.,* (2011) investigated the polyketide synthase gene Fum 5 region. They thought that this region has unique primer binding sites for distinguishing Fumonisin..

Several factors can affect in *vitro* production of fumonisin including limited nitrogen, pH of 5.9 levels and water activity. Miller, 2001 said that presence of mycotoxin gene only not enough to designate that the pathogen is completely potential to produce fumonisin since it lies in their expression in association with plant system. On the other hand, this gene identification leads to further studies like gene expression and level of fumonisin production at different stages of fungus. The molecular methods are rapid in detection of fumonisin producing from Fusarium species, as there is no need to culture organisms prior to their identification. In these molecular methods, the results are specific since they depend on the basis of genotypic differences and are highly sensitive, detecting the target DNA molecules in complex mixtures, even when the mycelia are no longer viable.

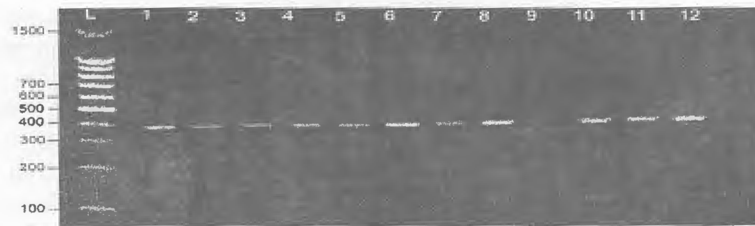

Fig. (1):Gel electrophoresis of 2% agarose gel showing 400 bp amplification fragment of the Inter Generic Spacer region (IGS) of *Fusarium*. Lane (1): DNA ladder, Lane (2): *F. verticillioides* (positive control), Lane (2-12): *Fusarlum moniliforme* except lane (9): *F. solani* (Negative control).

Fig. (2): Agarose gel electrophoresis showing fragment amplification of about 300 bp sizeof the FUM1 gene. Lane (1) DNA ladder, Lane (2): *F. verticillioides* (positive control) Lanes (2-12) *Fusarium moniliforme* except lane (9) *F. solani* (Negative control). Lane (11) exhibit different band (allele) 135 bp.

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تحديد سلالات الفيوزاريوم المنتجة للفيومنزين والمسببة لمرض البكانا في الارز باستخدام المعلمات الجزيئية.

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أجريت هذه الدراسة في مزرعة ومعمل مركز البحوث والتدريب في الارز بسخا كفر الشيخ خلال موسمين زراعيين ٢٠١٣، ٢٠١٤م وكان الهدف من الدراسة الاتي:
تحديد أى من العزلات الخاصة بفطر الفيوزاريوم وانواعة المختلفة الذي يصيب الارز والذرة والقمح القادرة على انتاج مادة الفيومنزين السامة.
الفيومنزين: عبارة عن سموم تنتج عن طريق فطر الفيوزاريوم وانواعة المختلفة سواء كان العائل الارز أو الذرة أو القمح ومن خلال الدراسات والأبحاث الأخيرة وجد أن هذه المادة تقوم بدور كبير في حدوث السرطانات المختلفة مثل سرطان المريء في الانسان بجنوب أفريقيا وايضا في الحيوانات التى تتغذي على هذه المحاصيل مثل التورم الرئوي واختلال المخ في الخيول.
فعن طريق منع دخول هذه المادة داخل سلاسل الغذاء يكون من الطرق الأولية لمكافحة هذه الأمراض وقد ظهر هذا المرض حديثاً في مصر فكان لابد من وضع خطة لتتبع هذا المرض أو الفطر ومعرفة اى السلالات تكون قادرة على انتاج الفيومنزين وفي أى محافظة عن طريق الاتي:
١. عن طريق عزل هذه المرض من ست محافظات(كفر الشيخ – الدقهلية – دمياط– البحيرة – الغربية) وقد تم عزل ١٢ عزلة من هذه المرض، وباستخدام PCR والمعلمات الجزيئية اثنين وباستخدام اثنين من specific primers مثل ١. VERTF-1 ، ٢. FUM1.
٢. تم تحديد أى من هذه العزلات قادرة على انتاج الفيومنزين عن طريق primer الاول (VERTF-1) وجد ان العزلة رقم ٩ من محافظة كفرالشيخ والمعزولة من القمح وباستخدام الدراسات المورفولوجية التى تم القيام بها سابقا وجد أ، هذا القطر هو Fusarium solani هذه العزلة غير قادرة على انتاج الفيومنزين
٣. وجد أن باستخدام ال Primer الثاني FUM1 فهو أكثر دقة وكفائة وتخصصا لان هذا البادئ او الجين تم تحديدة بدقة في الجين بنك N.C.B.I. ومعرفة التتابعات الخاصة به بدقة وتحديدا لاربع جينات تم التعرف التبايع النيوكلتيدى لها وهذه الجينات مسئولة عن انتاج مادة polyketide وهى تعتبر المكون الأساسي لمادة الفيومنزين السامة.
٤. وجد أن العزلات ١، ٤ ،٦، ٨، ١٢ تحتوى على التبايع النيوكلتيدى الذي تم التعرف علية في N.C.B.I. والخاص بانتاج polyketide الخاصة بتكوين مادة الفيومنزين السامة.