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Diagnosis of Phytopathogenic Fungi of the genus Fusarium in Cereals on the Territory of the Republic of Kazakhstan using the Quantitative PCR Method

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Abstract: Diagnosis of pathogenic species of fungi of the genus Fusarium has always been hampered by the confusing phenotypic classification system. We used a method based on quantitative PCR (real-time PCR) with primers and probes developed by our laboratory which allows to quickly and accurately identify three pathogenic species of Fusarium fungi common in Northern Kazakhstan: *F. graminearum*, *F. culmorum* and *F. oxysporum*. The method is based on PCR amplification of DNA fragments which were developed on the basis of the translation elongation factor gene 1-α (tef1α). In addition to providing accurate, reliable and rapid diagnosis of fungi of the genus fusarium in cereals, this method excludes the use of carcinogens (ethidium bromide). since, quantitative PCR uses fluorescent labeled probes, i.e., no gel electrophoresis is required.

INTRODUCTION

Food and biological safety in the world largely depends on a clear and timely control over the phytosanitary condition of the environment and agricultural plants as well as the products of their processing and feed. The main approach for such monitoring is the effective diagnosis and identification of phytopathogens. Particular attention should be paid to the diagnosis of pathogens that are objects of internal and external quarantine as well as especially dangerous pathogens.

Environmental conditions strongly affect the species composition of representatives of the genus Fusarium in this or that region. We can say that they are the key factor in the occurrence and prevalence of pathogens, since, Fusarium pathogens are not confined to any one species of host plant[1]. For a long time fusariosis was considered as a disease typical for areas with a warm and humid climate. Such, conditions are optimal for the development of fungus *F. graminearum* which caused the most famous epiphytoty of fusarium. However, later with the identification of new species, it became clear that many of them are characterized by high ecological plasticity and can occur in regions with different climatic conditions. The same *F. graminearum* in the early 2000s was first discovered in northern Europe (Norway, Finland, Poland, Northern Germany)[2] as well as in northwest Russia[3].

Apparently, this was favored by the warming of the climate as well as the development of mechanisms for adapting the fungus to colder conditions.

Pathogenic fungi of the genus Fusarium, produce harmful mycotoxins as a result of which these toxins contaminate crop production[4]. The use of food and fodder products contaminated with mycotoxins poses a great risk to human health and animals, since, these mycotoxins are carcinogens and can weaken the immune system[5]. Outbreaks of diseases caused by these fungi are a big problem for the agricultural industry and threaten global food security[6, 7]. To date, there is a need for precise and rapid control measures for diseases caused by these fungi, since, the precise identification of fungi of the species Fusarium has always been problematic even for experts in mycology.

At the moment, molecular technologies play an important role in studying the diversity of fungi of the genus Fusarium as well as in their species-specific identification[8]. From this perspective, approaches that are based either on comparing the spectra of specific metabolites of fungi (in particular toxins) or on the analysis of polymorphism of genomic and mitochondrial DNA are particularly relevant. The weak side of the first approach is that the absence of toxin does not mean the absence of their producer as the synthesis of toxin can begin when the storage conditions change, under the influence of biotic and abiotic stresses[9]. The most specific for the detection of fusariosis pathogens are methods using DNA markers, primarily based on Polymerase Chain Reaction (PCR) which allow to identify the species by the characteristic sequence of nucleotides of its DNA. In recent year, the use of molecular methods has made it possible to clarify the taxonomic status of isolates of fungi of the genus Fusarium of various origins[10-13] and also to isolate a number of new types of fusarium pathogens[14-16].

Recently, the wider use of molecular methods in the diagnosis of fungal diseases of plants is used as a possible solution to the problem associated with the existing phenotypic identification system[17, 18]. One of the most reliable and informative methods used in the diagnosis of fungal diseases is the PCR method.

The express method proposed by us can serve as a tool for assessing the level of fungal DNA content in cereals and assess the risk of mycotoxin contamination with fungi of the genus Fusarium.

MATERIALS AND METHODS

As a material for the study, strains of fungi found in the territory of northern Kazakhstan and grain samples infected with fungi of the genus Fusarium as well as herbarium material were used.

Isolation of DNA: To obtain a plentiful mycelium, the culture of the fungi was transferred to potato agar and grown in the dark for 10 day, *Fusarium graminearum* was cultivated on potato agar with dextrose at 25°C for 6 days. DNA isolation from fungal cultures and contaminated wheat seedlings was carried out using a commercial PureLink DNA extraction kit from the United States. The DNA extraction procedure was carried out in accordance with the manufacturer's instructions.

The DNA concentration was determined using a Halo DNA master Dynamica spectrophotometer (“Dynamica GmbH”, UK). Before introducing into the reaction mixture, the DNA isolated from the cultures was diluted to 10 ng mL^{-1}.

Selection of specific primers and fluorescently-labeled probes. The search for nucleotide sequences for the selection of specific primers was performed using GenBank NCBI online program (http://www.ncbi.nlm.nih.gov/GenBank). Alignment of nucleotide sequences was performed using the Clustal W algorithm. The efficiency of the primers and probes was checked using Oligo 6.71.

PCR and analysis of results: The amplification was carried out using the QuantStudio 5 Real-Time PCR System thermal cycler (“Applied Biosystems™”, USA). The composition of the reaction mixture consisted of: 18 μL of a 1.25× PCR buffer, 0.24 μL of 25 mMdNTPs, 0.125 μL of each primer (100 μM), 0.14 μL probe (50 μM), 10 μ Taq polymerase solution, 5 μLμL of DNA (all reagents produced by LLC Agrodiagnostika). A threshold analysis method was used to determine Cq values. Primers and fluorescently-labeled probes were synthesized in ZAO “Sintol” (Moscow).

PCR was performed in accordance with the following amplification programs: for primer pairs of the translation elongation factor 1-alpha (tef1α): 93°C-90 s; 93°C-20 s, 64°C-5 s, 67°C-5 s (5 cycles); 93°C-1 s, 64°-5 s, 67°C-5 s (40 cycles).

The probe was a TaqMan. The detection of PCR results was carried out by gel electrophoresis. Electrophoresis was performed at a current of 400 mA in a 2% agarose gel in TAE buffer (40 mMtris-hydroxymethylaminomethane, 20 mM glacial acetic acid, 1 mM EDTA). The molecular weight of the fragments was estimated using DNA markers with a molecular weight of 50 base pairs (GeneRuler 50 bp DNA ladder, Thermo Scientific). The results of electrophoresis were visualized on the QUANTUM gel model system model 1100 SUPER (Viber-Lourmat). Sequencing of DNA molecules was performed at ZAO Evrogen on an automatic sequencer ABI PRISM 3730 Applied Biosystems using the ABI PRISM® BigDye™ Terminator v. Reagent kit. 3.1 by the method of Sanger. Plasmids were used as positive controls and to calibrate the sensitivity of the reaction.

Table 1: List of oligonucleotides selected for identification of *Fusarium graminearum*, *F. culmorum* and *F. oxysporum*

Paraprimer	Object (s)	Gene	5'-3'sequence
Fgc210F	*F. graminearum*	*TEF1α*	CCCAACCCCGCCGACACT
Fgc605R			GGTTTGTGGGAAGAGGGCAGA
Fsl80F	*F. culmorum*	*TEF1α*	CCCAACCCCGCCGATACA
Fsl390R			GGTTTGTGGGAAGAGGGCAAG
FoxF	*F. oxysporum*	*TEF1α*	AGTACTCTCCTCGACAATGAGC
FoxR			TGAGTACTCTCCTCGACAATGAGC

Table 2: Structures of fluorescently-labeled probes (TaqMan) used in the work

Probe	Sequence 5'→3'	T_m°C	Specificity
FgcP(T)	BHQ1-GGGCTCA(FAMdT)ACCCCGCCACTCGAG	75	*F. graminearum, F. culmorum*
FoxP(T)	BHQ1)-TGACTTTGAGAAA(FAMdT)ACCCCGCCAGGTCTTG(p)	75	*F. oxysporum*

RESULTS AND DISCUSSION

Since, the most common loci used as targets in PCR diagnostics of phytopathogenic fungi are the "home" genes and ribosomal DNA segments (primarily the internal transcribed ITS spacer and the IGS intergenic spacer), ITS was chosen as the three potential targets and also the beta-tubulin genes and the translation elongation factor 1 alpha (tef1α). The most informative of these loci was the gene tef1α. In Table 1 shows the numbers under which partial sequences of the tef1α gene used in the selection of primers were deposited in GenBank NCBI.

For each pathogen, two pairs were initially selected, from which the most optimal was subsequently selected. The primers were selected in accordance with the following requirements:

- Length of primers-18-30 bp
- GC-composition of primers, determining Tm (melting point), should be within 40-60% (optimally 45-55%). In this case, the values of Tm of both primers should be close
- The terminal 3'-nucleotides must not be complementary to the primer itself, another primer of the pair, the probe or other synthetic nucleotides added to the reaction
- If the primers require specificity, then at least 2 nucleotides at the 3 'ends must be homologous to the DNA of the organism being analyzed and not homologous to the DNA of the closely related organisms
- It is desirable that the Tm of the 5' end of the primer exceeds the Tm of the 3'-terminal portion
- It is allowed to add to the 5'-end of several bases, non-complementary matrix

The next step was to select probes for fluorescent detection. The probe does not require such strict specificity as in the case of primers, so, it was possible to design universal probes to conserved sections of DNA nucleotide sequences. As a result, 2 probes were selected for work-one for the detection of *F. graminearum* and *F. culmorum*, one for the detection of *F. oxysporum* (Table 2).

Table 3: Threshold cycle values for consecutive dilutions of plasmid-positive controls. *F. gram-F. graminearum; F. culm-F. culmorum; F. oxysp-F. oxysporum*

Example	*F. gram.*	*F. culm.*	*F. ox.*
F. graminearum	+ (19.0)	-	-
F. culmorum	-	+ (18.7)	-
F. sporotrichioides	-	-	-
F. cerealis	-	-	-
F. poae	-	-	-
F. langsethiae	-	-	-
F. avenaceum	-	-	-
F. oxysporum	-	-	+ (26.3)

In all cases, the value of fluorescence of the probe exceeded by 2.5 times the level of its background fluorescence which indicates the absence of inhibition of PCR. In embodiments where the observed formation of a specific PCR product is less than that of the signal by several times compared to those in which the formation of a specific product does not occur. The DNA of one spore culture of Fusariumgraminearum, Fusariumculmorum, Fusariumoxysporum using quantitative PCR using primers for tef1α is shown in Table 1.

In Table 3 shows the amplification of DNA from monospore strains of Fusarium pathogens of various species. The values of the threshold cycles for each sample are given in parentheses. HEX-internal control (plasmid amplified by the principle of multiplex PCR, necessary to detect false negative results).

Analysis of the sensitivity and effectiveness of the developed test systems. To assess the sensibility and efficiency of the reaction, PCR-PB was performed on consecutive tenfold dilutions of the plasmids used as positive controls. Each concentration (in the range from 101-108 copies of DNA per reaction) was analyzed in four replicates. For all systems developed, the sensitivity was 100 copies of the specific DNA for the reaction. In Table 4 shows the values of the threshold cycles (Cq) for each of the systems developed.

Based on the results obtained, the amplification efficiency was calculated which was 88% for

Table 4: Threshold cycle values for consecutive dilutions of plasmid-positive controls. *F. gram-F. graminearum; F. culm-F. culmorum*; *F. oxysp-F. oxysporum*

Concentration	*F. gram.*	*F. culm.*	*F. oxy.*
10^8	6.8	6.1	7.80
10^7	9.5	9.0	10.7
10^6	13.0	12.5	14.4
10^5	16.2	15.8	17.0
10^4	19.0	18.8	21.0
10^3	23.1	22.2	25.7
10^2	27.2	26.2	29.0
10^1	-	-	-

F. graminearum, 90% for *F. culmorum*, 85% for *F. oxysporum*. These values are relatively high and indicative of correct PCR optimization and the effectiveness of the proposed diagnostic systems.

CONCLUSION

As a result of analysis of DNA nucleotide sequences deposited in the international database of GenBank NCBI, polymorphic loci suitable for selecting species-specific primers were selected, primers specific for the DNA of phytopathogenic fungi *F. graminearum*, *F. culmorum*, *F. oxysporum* were constructed; their specificity is shown experimentally in tests with DNA of monospaced cultures of fungi, PCR conditions are optimized-optimal annealing temperatures, temperature cycle parameters, duration of each amplification stage, systems are adapted to PCR format “in real time”, for which probes of TaqMan type for fluorescent detection, positive control samples for each of the test systems were constructed which were used to assess the sensitivity of the systems which amounted to about 100 copies of DNA but a reaction.

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REFERENCES

01. De Wolf, E.D., L.V. Madden and P.E. Lipps, 2003. Risk assessment models for wheat *Fusarium* head blight epidemics based on within-season weather data. Phytopathology, 93: 428-435.
02. Obst, A., B. Gunther, R. Beck, J. Lepschy and H. Tischner, 2002. Weather conditions conducive to *Gibberella zeae* and *Fusarium graminearum* head blight of wheat. J. Appl. Genet., 43: 185-192.
03. Gavrilova, O.P., T.Y. Gagkayeva, A.A. Burkin and G.P. Kononenko, 2009. Infection with fungi of the genus fusarium and contamination with mycotoxins of grain of oats and barley in the North of the non-black earth region. Agric. Biol., 6: 89-93.
04. Doohan, F.M., D.W. Parry, P. Jenkinson and P. Nicholson, 1956. The use of species specific PCR based assays to analyse fusarium ear blight of wheat. Plant Pathol., 47: 197-205.
05. Withanage, G.S., H. Murata, T. Koyama and I. Ishiwata, 2001. Agonistic and antagonistic effects of zearalenone an etrogenic mycotoxin, on SKN, HHUA, and HepG2 human cancer cell lines. Vet. Hum. Toxicol., 43: 6-10.
06. McMullen, M., R. Jones and D. Gallenberg, 1997. Scab of wheat and barley: A re-emerging disease of devastating impact. Plant Dis., 81: 1340-1348.
07. Clear, R.M., S.K. Patrick and D. Gaba, 2000. Prevalence of fungi and fusariotoxins on barley seed from western Canada, 1995 to 1997. Can. J. Plant Pathol., 22: 44-50.
08. Benali, S., B. Mohamed, H.J. Eddine and C. Neema, 2011. Advances of molecular markers application in plant pathology research. Eur. J. Sci. Res., 50: 110-123.
09. Doohan, F.M., J. Brennan and B.M. Cooke, 2003. Influence of climatic factors on *Fusarium* species pathogenic to cereals. Eur. J. Plant Pathol., 109: 755-768.
10. O'Donnell, K., 2000. Molecular phylogeny of the Nectria haematococca-*Fusarium solani* species complex. Mycologia, 92: 919-938.
11. Yli-Mattila, T., S. Paavanen-Huhtala, S.A. Bulat, I.A. Alekhina and H.I. Nirenberg, 2002. Molecular, morphological and phylogenetic analysis of the *Fusarium avenaceum*/*F. arthrosporioides*/*F. tricinctum* species complex-a polyphasic approach. Mycol. Res., 106: 655-669.
12. Obanor, F., G. Erginbas-Orakci, B. Tunali, J.M. Nicol and S. Chakraborty, 2010. *Fusarium culmorum* is a single phylogenetic species based on multilocus sequence analysis. Fungal Biol., 114: 753-765.
13. Harrow, S.A., R. Farrokhi-Nejad, A.R. Pitman, I.A.W. Scott, A. Bentley, C. Hide and M.G. Cromey, 2010. Characterisation of New Zealand Fusarium populations using a polyphasic approach differentiates the *F. avenaceum*/*F. acuminatum*/*F. tricinctum* species complex in cereal and grassland systems. Fungal Biol., 114: 293-311.
14. Scott, J.B. and S. Chakraborty, 2006. Multilocus sequence analysis of *Fusarium pseudograminearum* reveals a single phylogenetic species. Mycol. Res., 110: 1413-1425.

15. Kulik, T., A. Pszczółkowska and M. Lojko, 2011. Multilocus phylogenetics show high intraspecific variability within *Fusarium avenaceum*. Int. J. Mol. Sci., 12: 5626-5640.
16. Yli-Mattila, T., T. J.O. Ward, K. Donnell, R.H. Proctor, A.A. Burkin, G. P. Kononenko and T.Y. Gagkaeva, 2011. *Fusarium sibiricum* sp. nov, a novel type A trichothecene-producing Fusarium from northern Asia closely related to *F. sporotrichioides* and *F. langsethiae*. Int. J. Food Microbial., 147: 58-68.
17. Donaldson, G.C., L.A. Ball, P.E. Axelrood and N.L. Glass, 1995. Primer sets developed to amplify conserved genes from filamentous ascomycetes are useful in differentiating fusarium species associated with conifers. Appl. Environ. Microbiol., 61: 1331-1340.
18. Taylor, J.W., D.M. Geiser, A. Burt and V. Koufopanou, 1999. The evolutionary biology and population genetics underlying fungal strain typing. Clin. Microbial. Rev., 12: 126-146.