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Development of Sensitive High-Specific Rapid Tests Based on Dna Markers for the Diagnosis of Pathogenic Fungi of genera Puccinia and Pyrenophora, Causing Diseases of Cereals

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Abstract: Traditional diagnostic methods, such as enzyme-linked immunosorbent assays and microscopic diagnostics, require quite a lot of time do not have the necessary high sensitivity and do not always provide unambiguous identification of species. Therefore, the creation of test systems that allow the detection of pathogens quickly and accurately remains an urgent task. The Polymerase Chain Reaction (PCR) method is more sensitive and specific in comparison with microbiological methods and its real-time modification (quantitative PCR) using a hybridization probe with a fluorescent label makes it possible to quickly analyze the results without performing electrophoresis.

INTRODUCTION

On the territory of the Republic of Kazakhstan as in other countries engaged in the production of grain products, diseases caused by fungi *Pyrenophora tritici-repentis*, *Puccinia graminis* and *Puccinia recondita* are among the most dangerous diseases of cereals.

To date, over 100,000 species of fungi are known, among which there are many pathogenic, causing diseases of plants, animals and humans. According to modern estimates, fungi are the most extensive group of organisms that cause plant diseases. They are also the only group of pathogenic organisms in which all groups of parasitic specialization are represented: from necrothrophy to Biotrophs. The total damage caused by fungal diseases of cultivated plants worldwide is estimated at billions of dollars each year. Thus, according to the data of the Food and Agriculture Organization of the United Nations (FAO), in the last years of the 20th century, losses of wheat amounted to 33.5 million tons, i.e., about 10% of the potential harvest. Epiphytoty of fungal diseases and today can cause hunger, cause significant damage to both developing and developed countries.

The problem of contamination of crops with phytopathogenic fungi is particularly acute for the countries of Central Asia as this region, according to FAO is characterized by the highest level of consumption of bread per capita (>200 kg year^{-1}) and agriculture is considered as one of the main factors of the economy . At the same time, Kazakhstan is a pronounced leader in the region in terms of production (9.6 million tonnes per year in 2012) and exports of wheat and has the most extensive arable land in the region (82.4% of the total area).

Ensuring the quality and safety of food and raw materials used for their production requires a constant monitoring of the contamination of plants and grain with phytopathogens including rust and spotting agents and the development of effective methods for combating diseases, for example, the creation of resistant plant varieties. These methods should be based on integrative approaches that take into account all aspects of the disease but effective, first of all, accurate, reliable and timely diagnostics and identification of pathogens are needed.

Rust and spotting of wheat are one of the most widespread and harmful diseases affecting cereals in the territory of Kazakhstan. Over the past 15 years, a number of outbreaks of epiphytoty in the region have been noted[1]. Well-known epiphytoty of stem rust in the Northern regions of Kazakhstan in 1967 which covered >5 million hectares of crops, resulting in crop losses exceeded 50%[2, 3]. The causative agents of brown (leaf) and linear (cauline) rust of wheat (*Puccinia recondita* and *Puccinia graminis*, respectively) are referred to basidiomycetes (class Basidiomycetes) and the causative agent of yellow spot (pyrenophorosis) of wheat (*Pyrenophora tritici-repentis*) to marsupials (class Ascomycetes).

Puccinia recondita and *Puccinia graminis* are obligate two-farm parasites with a complete life cycle and several types of sporulation. In the composition of these species a large number of races (pathotypes) are distinguished, differing by aggressiveness and virulence towards certain varieties. Intermediate hosts play an important role in the variability and maintenance of populations of rust pathogens-for example, weed plants such as the widely distributed northern forest hazel (*Isopyrum fumaroides*). Stem rust spreads rapidly over long distances with the help of wind or through transmission through a person. In recent years, researchers have focused on the highly aggressive race of *P. graminis* Ug99, first discovered in 1999 in Uganda[4]. There is a serious danger of its spread to the countries of central Asia, including Kazakhstan. In this connection, constant control of wheat contamination by rust pathogens is necessary, based on the application of fast and reliable modern methods of analysis.

The stem rust of wheat (*Puccinia graminis*), also known as black rust, is found everywhere where its hosts-wheat and other cereals-grow.

Epiphytotia of stem rust caused and cause a huge loss of wheat yield. A number of known cases of epiphytoty of this disease have been described in the literature. So, in 1935 in the states of Minnesota, North and South Dakota (USA), the disease claimed 50% of the wheat crop. According to Lelly, in 1963-1964. in Australia, 25% of the crop was killed (reference). In 1958 *epiphytoty* was noted in Europe (Czechoslovakia, Yugoslavia, Italy, etc.)[5]. The defeat of stem rust usually appears primarily on the stems and interstices of plants. Later it also affects the vagina, leaves, spikelets and even awns. The disease manifests itself in the spring in the form of brownish-red elongated pustules. On the edges of the pustule are visible the remains of the epidermis which burst under the pressure of the resulting spores of the pathogen. The brownish-red coloration of the pustules is attached to the accumulated mass of uredospores. Spore formation takes place on both sides of the leaves. As maturing, the pustules become black from the mass appearance of telithospores firmly attached to the tissues of the host. Hence the second name appeared-black rust[6].

Sheet rust (*Puccinia recondita*), called orange rust in many parts of the world in Europe-brown rust is also common in all regions where wheat is grown. The development of the disease depends on the weather conditions: it can appear as an epiphytoty either every year or at large intervals of time. The fungus is less sensitive to fluctuations in temperature, therefore, it is more widespread than stem rust.

Leaf rust causes noticeable crop losses: In the USA-20-25%, in the CIS countries, incl. in the Republic of Kazakhstan-4-5%. The most significant losses are noted in those cases when infections begin to develop from autumn. One hundred percent infection of plants in the tubing phase which persists to full ripeness of wheat can lead to a loss of up to 70% of the crop; If the infection occurs in the phase of milky wax ripeness, the losses can be 4-5%.

The yellow spot of wheat leaves, or pyrenephorosis, is caused by the homogeneous ascomycete *Pyrenophora tritici-repentis* which affects >60 species of fodder and wild-growing cereals. The loss of grain in epiphytotic diseases can reach 65%. Also an important feature of the fungus *Pyrenophora tritici-repentis* is the ability to produce a host-specific toxin (Ptr-toxin), responsible for the development of the necrotic reaction of the plant.

***Pyrenophora tritici-repentis* causes two types of cereal diseases:** Pyrenoforosis (yellow spot)-the disease occurs on wheat and some wild cereals. During the vegetation, the pathogen is spread by conidia by airborne droplets.

The disease is widespread but its diagnosis is fraught with difficulties. symptoms of pyrenephorosis resemble an atypical septoria. The disease appears on both sides of leaves and leaf sheaths of winter wheat and other cereal crops in the form of small single or multiple spots of oval or round shape, yellow or light brown in color with a diameter of 2-5 mm. The spots can be in the form of strips, occupying a third or even more than half of the leaf surface. The fungus can cause damage to spikelet scales. On them the spots are elongated or in the form of strokes 2-4×1-2 mm, not expanding.

Helminthosporiosis (ordinary rot of roots) affects seeds, shoots, roots and tissues of the bases of wheat stalks. The fungus usually inhabits the roots and the root

collar as a result of which the affected areas rot and rot. In addition to the roots and root collar, the lower part of the stem and the coleoptile also become brown. Later, the straw and the spike are sick which leads to a complete loss of grain in the ear or to its feel. The fungus can also attack ears and grain. Disease of the grain manifests itself in the form of the so-called "black germ" or hidden infection which is detected only when germinating grain[7].

Molecular genetic methods for studying cereal crop diseases are relevant and conducted around the world, for example, in the United States of America, studies were carried out to identify and map the fungi-pathogens *Pyrenophora tritici-repentis* and *Puccinia graminis*[8, 9], a group of scientists from Germany conducted studies to create a two-level molecular detection method for *Pyrenophora tritici-repentis*[10].

A brief analysis of the sequences of nucleotides of the causative agents of rust and wheat spotting deposited in the GenBank NCBI allows to conclude that the ribosomal DNA (rDNA) regions are most well characterized from the point of view of the structure. Most of the previously published phylogenetic studies of the genera Puccinia and Pyrenophora were based on the use of rDNA, primarily characterized by a relatively high polymorphism of the internal transcribed spacer 1 (BTS1). In particular, a system of PCR detection of four main rust pathogens (*P. graminis*, *P. striiformis*, *P. reconditaf* sp. secalis and *P. reconditaf.* sp. tritici[9] was proposed. The specificity of the developed system was based on the use of four fluorescent-labeled probes such as TaqMan as well as one pair of primers which amplified fragments of different sizes depending on the type of pathogen. However, from our point of view, the possibility of using the described system in the routine diagnosis of rust pathogens is limited, due to several factors. First, it is desirable that the specificity of the test system be determined by primers and not by probes-this approach reduces the number of tests that must be performed to characterize the sample. To select a specific probe, it is necessary that the DNA sequences of closely related species have significant differences that would ensure that the probe is not annealed while to ensure the primer specificity, a 1-2 nucleotide difference in the sequence corresponding to its 3 'end is sufficient. Provided that the primers are strictly specific, it is possible to select one universal probe which will reduce the financial and time costs of synthesis. In addition in the present work, the DNA amplification products of four rust pathogens vary in size from 260-270 bp which makes it practically impossible to distinguish amplicons by electrophoresis. Thus, the proposed system is of scientific interest but its use in the practice of diagnosing plant material with phytopathogenic fungi is difficult.

RAPD, AFLP as well as microsatellite analysis are used for genotyping and identification of rust pathogens. The application of these approaches makes it possible to assess the degree of genetic polymorphism at the inter and intraspecific level but it is almost impossible to apply them for routine diagnosis.

These same approaches have found their application for genetic studies of the causative agent of yellow spot of wheat[11, 12]. In addition, polymorphic markers were subsequently identified using AFLP analysis which were subsequently used to select PCR primers that provide specific identification of the pathogen of barley spotting (Pyrenophorateres)[13].

Thus, to date not a single system has been described that provides a fast and unambiguous identification of the investigated pathogens of rust and wheat spot, therefore, the proposed development is new both in Kazakhstan and in the world as a whole. Achieving the goals and solving the problems formulated in this project is relevant for the country's food and economic security, since, it allows to significantly improve the quality of plant material and its processing products through effective continuous monitoring for contamination with phytopathogenic fungi. The use of the proposed test systems makes it possible to significantly accelerate the mass screening of samples of cultivated plants and grains. In addition, the proposed test systems will find their application in the study of the dynamics of infection and the analysis of the effect of fungicides.

Today's state of the art allows the effective implementation of the proposed test systems in production and routine diagnostic practice. The equipment necessary for each of the stages of development and introduction of tests is available: synthesizers for the preparation of oligonucleotide primers and probes; amplifiers and PCR detectors "in real time", equipment for electrophoretic analysis, appropriate computer programs for processing the results of analysis.

MATERIALS AND METHODS

The object of the study was monospore cultures of fungi and samples of plants infected with *Puccinia graminis*, *Puccinia recondite*, *Pyrenophora tritici-repentis*.

Isolation of DNA: To obtain a plentiful mycelium, the culture of Puccinia genus was transferred to potato agar (PDA) and grown at 25°C for 6 days, *Pyrenophora tritici-repentis* was grown on oat (Oat agar) and vegetable agar (V8) at 22°C. DNA extraction from cultures of fungi and infected wheat seedlings was carried out using a commercial DNA extraction kit "PureLink" (manufacturer, USA). The procedure for DNA isolation was performed according to the instructions attached to the DNA extraction kit "PureLink".

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

Design of specific primers and fluorescently-labeled probes. The search for nucleotide sequences for the selection of specific primers and probes was performed in the GenBank NCBI database (http://www.ncbi.nlm.nih.gov/GenBank). Alignment of nucleotide sequences was performed using the Vector NTI Advance program suite. The physico-chemical properties of the primers and probes were verified using the Oligo 6.71 program.

PCR and analysis of results: The amplification was carried out using the QuantStudio 5 Real-Time PCR System thermal cycler ("Applied Biosystems™", USA). In the amplification mixture of the following composition: 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 μLTaq polymerase solution, 5 μL of the DNA solution (All reagents produced by OOO Agrodiagnostika). The reaction was carried out in accordance with the following amplification profile: 93°C-90 s; 93°C-20 s, 64°C-5 s, (5 cycles); 93°C-1 s, 64°-15 s, (40 cycles). A threshold analysis method was used to determine Cq values. Primers and fluorescently-labeled probes were synthesized in OOO Agrodiagnostika (Moscow).

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 2.5% agarose gel in TAE buffer (40 mMtris-hydroxymethyl aminomethane, 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 bpdna ladder, Thermo Scientific). The results of electrophoresis were visualized on the QUANTUM gel filtration system model 1100 SUPER, VilberLourmat. 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.

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). In our work, the sequences of internal transcribed ribosomal DNA spacers (ITS1-2) proved to be the most suitable for the design of specific primers (Table 1 and 2).

In all cases, the value of the fluorescence of the IC 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 the VC signal several times compared to variants in which the formation of a specific product does not occur. The DNA of one S. tritici and S. nodorum spore culture using quantitative PCR using primers for the beta-tubulin gene and ITS are shown in Table 3.

In some samples there is no signal from internal control, apparently, this may be due to competitive inhibition of a specific sample. Thus, none of the pairs of primers showed a cross-reaction with other related species.

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 to 108 copies of DNA per reaction) was analyzed in four replicates. For systems specific to *P. graminis* and *P. recondite*, the sensitivity threshold was 100 copies, for a system specific for *P. tritici-repentis*, 1000 copies. In Table 4 shows the values of the threshold cycles (Cq) for each of the systems developed.

Table 1: Structures of oligonucleotide primers used for specific detection of three pathogenic fungi

Pairofprimers	Object (s)	Gene	5'-3'sequence
Pgram	*P. graminis*	ITS	GGATGTTGAGTGTTGCTGTACC
			TTGGGTTTTAGGAGTCTCTTATTAAC
Prec	*P. recondita*	ITS	AGATCATTGTGATTAAGTATACGTAAT
			GTATGGTTCTTTAGAAGTCTCTTTC
Ptr	*P. tritici-repentis*	ITS	CTGGACAAGAGCGCAAATAATG
			CCGCCAATTGGACCTTATTC

Table 2: List of selected probes

Types	Locus (access code in GenBank)	Sequence, 5'-3'	Length
Puccinia graminis	ITS1-ITS2 (AF468044.1)	(BHQ1)-AAAGGTGCAAGA(FAMdT)GCGTTCAAAGATTCGAT	30
Puccinia recondita	ITS1-ITS2(EU014045.1)	(BHQ1)-TCGAATCTTTGA(FAMdT)ACGCACATTGCGC	26
Pyrenophora tritici-repentis	ITS1-ITS2(AM887495.1)	(BHQ1)-GCTTGGTGT(FAMdT)GGGCGTCTTGTCTCTCTCCC	30

Table 3: Results of DNA amplification in "real time" mode

Examples	*P.gram*	*P.rec.*	*P.tr.*
P. graminis (monosp. to-ra)	+(19.0)	-	-
P. recondita (monosp. to-ra)	-	+(18.7)	-
P. tritici-repentis (monosp. to-ra)	-	-	+(18.3)
Infected plant No.1	+(25.0)	+(22.7)	-
Infected plant No.2	+(22.3)	+(27.0)	+(25.7)
Infected plant No.3	+(24.7)	-	+(26.0)
Infected plant No.4	+(25.1)	+(28.5)	+(28.2)
Infected plant No.5	+(24.2)	-	+(26.3)
Infected plant No.6	+(23.9)	+(27.3)	-

Table 4: Threshold cycle values for consecutive dilutions of plasmid-positive controls. *P. gram-P. graminis; P. rec-P. recondita; P. tri-rep - P. tritici-repentis*

Concentration	*P. gram.*	*P. rec.*	*P. tri-rep.*
10^8	7.0	6.5	8.9
10^7	11.0	10.2	13.1
10^6	13.5	13.5	17.0
10^5	16.5	16.8	20.5
10^4	19.9	20.1	24.0
10^3	23.3	23.4	27.3
10^2	27.1	26.8	-
10^1	-	-	-

Based on the results obtained, the amplification efficiency was calculated which was 96% for *P. recondite*, 92% for *P. graminis*, 91% for *P. tritici-repentis*. These values are relatively high and indicative of correct PCR optimization and the effectiveness of the proposed diagnostic systems.

CONCLUSION

As a result, we developed a test system that allows to detect pathogenic fungi *Pyrenophora tritici-repentis*, *Pucciniag raminis* and Puccinia recondite, these fungi are widespread in the regions engaged in the cultivation of cereal crops, including in Northern Kazakhstan. The test system operates in the quantitative PCR mode which excludes from the diagnostic process the electrophoresis stage, i.e., does not require the use of carcinogens which are used for electrophoretic separation of PCR products. For each of the test systems, positive control samples were constructed which were used to assess the sensitivity of the systems which was from 100-1000 copies of DNA per reaction.

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