IRSTI 68.37.31; 34.15.23 https://doi.org/10.26577/ijbch.2022.v15.i1.07

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(Received 15 March 2022; received in revised form 19 April 2022; accepted 28 April 2022)

Identification of wheat samples for resistance to toxins of *Pyrenophora tritici-repentis* (Ptr)

Abstract. *Pyrenophora tritici-repentis* (Ptr) is a causal agent of tan spot in wheat in Kazakhstan, as it has been around the world. The pathogen produces host-specific toxins which interact with the wheat host sensitivity loci. The aim of this study was 1) to identify whether selected Kazakhstani isolates of *P. tritici-repentis* possessed the Ptr toxin genes *ToxA* and/or *ToxB* and 2) to identify the wheat varieties resistant to HST ToxA and ToxB. As a result of the analysis of the frequency of occurrence of PTR races, it was found that races 7 (25%) and 8 (41.6%) dominate in isolates from southern Kazakhstan, and race 4 (62.5%) prevails in northern Kazakhstan. Twenty single spore isolates collected from wheat-growing areas of the South and North of Kazakhstan representing the *P. tritici-repentis* population were characterized for the presence of the *Ptr ToxA* and *Ptr ToxB* genes, using two gene specific primers. Eight (40%) Kazakhstani *P. tritici-repentis* isolates were positive for the *ToxA* gene, and two isolates (10%) were positive for the *ToxB* gene. *ToxB* gene was not previously found in our country, but the results of this study show the appearance of this toxin in south Kazakhstan. Eleven (64.7%) wheat varieties resistant to HST ToxA were identified using molecular markers linked to the *tsn1* gene, insensitive to Ptr ToxA. The identified genotypes are recommended for use in breeding for wheat resistance to tan spot.
Key words: *Pyrenophora tritici-repentis*, isolates, host-selective toxins, race, tan spot.

Introduction

Tan spot caused by *Pyrenophora (P.) tritici-repentis* (syn. *Drechslera tritici-repentis* (Died.) Shoemaker), is an economically important foliar disease of wheat (*Triticum* spp.) worldwide, including Australia, Europe, USA, Canada and South America [1, 2], Russia [3-4]. In Kazakhstan, every year the spectrum of pathogens is growing, which significantly reduce the wheat yield. These pathogens include *Puccinia graminis* f. sp. *Tritici* [5-7], *Puccinia striiformis* West end. f. sp. *tritici* [8], *Puccinia triticina Erikss.* [9], *Tilletia caries* [10]. In recent years there has been increasing distribution and harmfulness of *P. tritici-repentis* in Kazakhstan [11-16].

Tan spot infection can result in two distinct symptoms, necrosis (tan colour) and extensive chlorosis (yellow colour). On leaves, the lesions characteristically have small tan/ brown centres, surrounded by a yellow circular border. As the plant matures, *P. tritici-repentis* (Ptr) infects the stem where it will begin to develop pseudothecia [17].

The development of the different characteristic symptoms is highly specific and a result of an interaction between host-selective toxins (HST) secreted by the pathogen and the target receptors of a toxin-sensitive host wheat plant [18, 19]. Three HST (Ptr ToxA, Ptr ToxB and Ptr ToxC) have been characterized to date. Both Ptr ToxA, which induces necrosis on susceptible wheat genotypes, and Ptr ToxB, which induces chlorosis on susceptible wheat cultivars, are proteinaceous in nature and are encoded, respectively by the genes *ToxA* [22-24] and a number of multi-copy genes such as *ToxB* [25-27]. At present, it is possible to screen for the presence of both genes *ToxA* and *ToxB* using specific molecular primers [28]. In contrast, although Ptr ToxC, which can induce chlorosis on specific wheat genotypes, has been suggested to be a non-ionic polar molecule,

its exact nature and the gene(s) encoding it have not been identified [27]. Additionally, there are also two other, uncharacterized HST, known as Ptr ToxD toxins, whose exact targets and functions have yet to be elucidated [28]. Studies of *P. tritici-repentis* populations in the USA, Canada, South America, Australia, the Baltic States and Romania have shown that Ptr ToxA has been the predominant HST found in these populations, with Ptr ToxB almost completely absent [19, 22, 26, 29].

Based on the ability of isolates to produce the different HSTs (and thereby necrosis or chlorosis) on a set of differential wheat cultivars, currently eight races of *P. tritici-repentis* have been identified worldwide [30]. However, there is little information on the presence of Ptr ToxA and/ or Ptr ToxB in populations of Kazakhstan *P. tritici-repentis* isolates.

An integrated plant disease control requires a combination of several tools to effectively combat the disease. In the case of tan spot, the use of resistant wheat varieties is the best option to sustainably manage the disease. In addition, it is the most cost-effective and environmentally friendly method of disease control. To this end, the breeding of resistant wheat varieties should be one of the main objectives of the tan spot control strategy, which should include assessment of germplasm diseases [31]. The aim of this study was 1) to identify whether selected Kazakhstani isolates of *P. tritici- repentis* possessed the Ptr toxin genes *ToxA* and/or *ToxB* and 2) to identify the wheat varieties resistant to HST ToxA and ToxB.

Materials and methods

In 2020, a collection of 113 single-pore isolates of *P. tritici-repentis* was created, of which 20 isolates were selected for further study.

Detection of Ptr-ToxA and Ptr-ToxB using PCR. The 20 Kazakhstani *P. tritici-repentis* single-spore isolates were screened using PCR for the presence of the Ptr ToxA and Ptr ToxB genes using ToxA and ToxB specific primers, respectively. The genomic DNA of 2 Ptr isolates, used as a control known to be either positive or negative for Ptr ToxA and Ptr ToxB, and from a range of different Ptr races, were obtained to validate the PCR and for comparison against the local Kazkhstani Ptr isolates. Genomic DNA for the 2 international *Ptr* isolates (control) from different geographic origin (Pskov and Greece 9) were provided by Dr Mironenko (All-Russian Research Institute of Plant Protection, St. Petersburg, Russia).

Genomic DNA extraction from P. tritici-repentis. The isolates of Ptr were grown for 3 weeks in liquid Fries medium amended with 1.5% yeast extract [30]. The mycelial mats were used for genomic DNA extraction. Briefly, 40 mg of lyophilized mycelium from each isolate was extracted with a Wizard® Genomic DNA Extraction Kit (Promega Corp, Madison, WI) in accordance with the protocol for plant material of the manufacturer. After these two extractions with phenol-chloroform (1:1 v/v) followed by one extraction with chloroform were performed. DNA concentration was measured using a NanoDrop-ND-1000 spectrophotometer (NanoDrop Technologies, United States of America) and DNA concentrations were diluted to 10–20 ng/μL for PCR. [19].

The Ptr isolates were screened for the presence of either the *ToxA* and *ToxB* genes using Ptr ToxA and Ptr ToxB gene-specific primers as described by Antoni et al. [26]. The forward (F) and reverse (R) primers for ToxA were: TA51F (5′-GCGTTCTATCCTCGTACTTC-3′) and TA52R (′5-GCATTCTCCAATTTTCACG-3′); and the *ToxB* primers were TB71F (5′-GCTACTTGCTGTGGCTATC-3′) and TB60R (5′-ACTAACAACGTCCTCCACTTTG-3′). Primer for *CHS-1* [31], the gene for chitin synthase, were included as an internal control for fungal DNA and produced a 275-bp amplification product (Table1).

Each PCR reaction volume was made up to 25 μL containing 1xPCR buffer (Roche), 200 μM dNTPs, 10 μM primer, 1 U FastStart Taq polymerase (Roche) and 1 μL template DNA. A negative control with 1 μL sterile water instead of the template DNA was included. A 7–10 μL aliquot of each PCR product combined with 3 μL loading dye was separated by electrophoresis at 10V/cm for 50 min in a 1% agarose gel (Bioline USA Inc.) alongside the 1 Kb plus DNA Ladder (Invitrogen™, Thermo Fisher Scientific Inc., USA). Gels were stained in ethidium bromide solution and visualised on a UV transilluminator (UVItec Cambridge Imaging System, Total Lab Systems Ltd). The presence of a band with the expected size for each gene indicated the presence the Tox gene. [34].

Identification of carriers of wheat varieties resistant to Ptr ToxA and Ptr ToxB. Genomic DNA extracted at two-leaf seedling stage for each individual plant using the CTAB method [19]. DNA concentration measured using a spectrophotometer SmartSpecTMPlus (Bio RAD). The DNA concentration for each sample was adjusted to 30 ng/μl. Samples were genotyped using the SSR marker *Xfcp623* designed to detect alleles of the

Tsn1 gene. The sequence of primers and PCR reaction conditions are given by [35]. The carriers of the *Tsn1* gene were also were detected using PCR protocol for SSR marker *Xfcp623* published at the WheatCAP website http://maswheat.ucdavis.edu/protocols]. The amplification products were separated on 2%-agarose gels, to determine the length of the amplification fragment 100 bp DNA Ladder (Ferments, Lithuania) was used. Gels were visualized on Gel Documentation System (Gel Doc XR+, BIO-RAD, Hercules, USA) for documentation of allele types in cultivars. Wheat entries Salamouni and Glenlea served as positive and negative controls, respectively [36].

Table 1 – Primers used for amplifcation of the Ptr ToxA and Ptr ToxB genes in *P. tritici-repentis* isolates

Gene	Primer for singleplex PCR	Sequence	Estimated band size (bp)	Reference
ToxA	TA51F TA52R	5′-GCATTCTCCAATTTTCACG-3 5′-GCTACTTGCTGTGGCTATC-3	573	[32]
ToxB	TB71F TB60R	5′-GCTACTTGCTGTGGCTATC-3 5′-ACTAACAACGTCCTCCACTTTG-3′	232	[32], [33]
CHS-1	CHS-79F CHS-354R	5′-TGGGGCAAGGATGCTTGGAAGAAG-3′ 5′-TGGAAGAACCATCTGTGAGAGTTG-3′	275	[32]

Results and discussion

P. tritici-repentis is a necrotrophic pathogen and a well characterized producer of host-specific toxin effectors. The results of previous studies have shown that, in 2016, it was shown that races 1 and 8 were dominant in Kazakhstan [37, 38]. Monosporic isolates of *P. tritici-repentis* isolated from the southeastern region in 2020 were assigned to certain races based on the manifestation of symptoms of necrosis/chlorosis on standard differentials (Glenlea, 6B662, 6B365).

In this study, a virulence was determined within the study of 113 single-spore isolates, which were isolated from infectious wheat material collected in the different regions of Kazakhstan during 2020 growing season. A total of 20 single spore Ptr isolates were recovered and characterized in this study (Table 2). Analysis of virulence of isolates from southern Kazakhstan allowed to determine the presence of five races: 4 (8.3%), 5 (16.6%), 6 (8.3%), 7 (50%) and 8 (16.6%). Isolates from northern Kazakhstan are determined by virulence to three races, including race 2 (12.5%), race 4 (62.5%), and race 7(25%).

Earlier five races of *P. tritici-repentis* have been identified, including races 1, 2, 3, 7 and 8; it has been shown that races 1 and 8 of *P. tritici-repentis* are dominant [38, 39]. As a result of current research, it was found that races 7 (25%) and 8 (41.6%) are the dominating in isolates from southern Kazakhstan, and race 4 is prevailing in northern Kazakhstan.

Table 2 – Molecular screening of Kazakhstan isolates of *P. tiritici-repentis* for the presence of *ToxA* and *ToxB, CHS-1* genes

*Isolate code	Race	PCR reaction			Region/Country collected
		ToxA	*ToxB*	*CHS1*	
KAZ-S-1-2021	7	-	-	275 bp	Almaty oblast, Kazakhstan
KAZ-S-2-2021	7	573 bp	-	275 bp	Almaty oblast, Kazakhstan
KAZ-S-3-2021	5	-	232 bp	275 bp	Almaty oblast, Kazakhstan
KAZ-S-4-2021	6	573 bp	-	275 bp	Almaty oblast, Kazakhstan
KAZ-S-5-2021	4	-	-	275 bp	Almaty oblast, Kazakhstan
KAZ-S-6-2021	7	-	-	275 bp	Almaty oblast, Kazakhstan
KAZ-S-7-2021	7	-	-	275 bp	Almaty oblast, Kazakhstan
KAZ-S-8-2021	8	573 bp		275 bp	Almaty oblast, Kazakhstan
KAZ-S-9-2021	7	-	-	275 bp	Almaty oblast, Kazakhstan

Continuation of the table

*Isolate code	Race	PCR reaction			Region/Country collected
		ToxA	*ToxB*	*CHS1*	
KAZ-S-10-2021	5	-	232 bp	275 bp	Almaty oblast, Kazakhstan
KAZ-S-11-2021	8	573 bp	-	275 bp	Almaty oblast, Kazakhstan
KAZ-S-12-2021	8	573 bp	-	275 bp	Almaty oblast, Kazakhstan
KAZ-N -1-2021	4	-	-	275 bp	Kostanay oblast, Kazakhstan
KAZ-N -2-2021	4	-	-	275 bp	Kostanay oblast, Kazakhstan
KAZ-N -3-2021	7	-	-	275 bp	Kostanay oblast, Kazakhstan
KAZ-N -4-2021	7	-	-	275 bp	Kostanay oblast, Kazakhstan
KAZ-N -5-2021	4	-	-	275 bp	Kostanay oblast, Kazakhstan
KAZ-N -6-2021	4	-	-	275 bp	Kostanay oblast, Kazakhstan
KAZ-N -7-2021	2	573 bp		275 bp	Kostanay oblast, Kazakhstan
KAZ-N -8-2021	4	573 bp		275 bp	Kostanay oblast, Kazakhstan
Spb-Pskov (Control for ToxA gene)	1	573 bp	-	275 bp	St. Petersburg, Russia
Spb-Greece 9 (Control for ToxB gene)	5	-	232 bp	275 bp	St. Petersburg, Russia
Notes: *Isolates designation: KZ for Kazakhstan, the number after the KAZ, indicates the number of the field, N or S is the southern or northern region from which isolates was collected, and the number after the dashed line is denoted for the particular isolates included in this study					

Using molecular markers detection of Ptr ToxA and Ptr ToxB was carried out. To control the ability to amplification of isolates DNA the primers for *CHS1* gene (the gene for chitin synthase) were used. It was found that CHS1 gene amplified a 275-bp amplification product from all isolates tested (Table 2). The Ptr ToxA specific primers amplified a band of 573 bp from the genomic DNA of 8 (40%) of the *P. tritici-repentis* isolates (KAZ-S-2-2021, KAZ-S-4-2021, KAZ-S-8-2021, KAZ-S-1-2021, KAZ-S-11-2021, KAZ-S-1-2021, KAZ-N-7-2021, KAZ-N-8-2021 and KAZ-S-12-2021). No amplification products of 573 bp were found in 13 isolates (KAZ-S-1-2021, KAZ-S-3-2021, KAZ-S-5-2021, KAZ-S-6-2021, KAZ-S-7-2021, KAZ-S-9-2021, KAZ-S-10-2021, KAZ-N-1-2021, KAZ-N-2-2021, KAZ-N-3-2021, KAZ-N-4-2021, KAZ-N-5-2021, KAZ-N-6-2021) indicating the absence of toxin ToxA, which accounted for 65% of the number of isolates studied.

Figure 1 – PCR amplification assays with primer for *ToxA* gene. Note: Lane: M, DNA ladder; 1, KAZ-S-1-2021; 2, KAZ-S-3-2021; 3, KAZ-S-2-2021; 4, KAZ-S-4-2021; 5, KAZ-S-5-2021; 6, KAZ-S-6-2021; 7, KAZ-S-7-2021; 8, KAZ-S-8-2021; 9, KAZ-S-9-2021; 10, KAZ-S-10-2021; 11, KAZ-S-11-2021, 12, KAZ-S-12-2021; 13, KAZ-N -1-2021; 14, KAZ-N -2-2021; 15, KAZ-N -3-2021; 16, KAZ-N -4-2021; 17, KAZ-N -5-2021; 18, KAZ-N -7-2021; 19, KAZ-N -8-2021, 20, KAZ-N -6-2021; 21, Pskov (positive control for *ToxA* gene); 22, ddH_2O (negative control for *ToxA* gene), M, DNA ladder

The ToxB specific primers amplified a band of 232 bp from the genomic DNA of the *P. tritici-repentis* isolates KAZ-S-3-2021 (Race 5) and KAZ-S-10-2021 (Race 5). A band of 232 bp were also observed in Greece 9, the reference positive control for ToxB gene (Figure 2). No PCR product was amplified using the ToxB specific primers in the 18 tested isolates of *P. tritici-repentis* (KAZ-S-1-2021, KAZ-S-2-2021, KAZ-S-4-2021, KAZ-S-5-2021, KAZ-S-6-2021, KAZ-S-7-2021, KAZ-S-8-2021, KAZ-S-9-2021, KAZ-S-11-2021, KAZ-S-12-2021, KAZ-N-1-2021, KAZ-N-2-2021, KAZ-N -3-2021, KAZ-N -4-2021, KAZ-N -5-2021, KAZ-N -6-2021, KAZ-N -7-2021, KAZ-N -8-2021).

The results of genotyping of 17 wheat entries with marker *Xfcp623* are given in Table 3. Of the tested 17 samples, 11 (64.7%) varieties and promising lines with insensitivity to Ptr ToxA were identified.

Figure 2 – PCR amplification assays with primer for *ToxB* gene. Note: Lane: M, DNA ladder; 1, KAZ-S-1-2021; 2, KAZ-S-2-2021; 3, KAZ-S-3-2021; 4, KAZ-S-4-2021; 5, KAZ-S-5-2021; 6, KAZ-S-6-2021; 7, KAZ-S-7-2021; 8, KAZ-S-8-2021; M, DNA ladder; 9, KAZ-S-9-2021; 10, KAZ-S-10-2021; 11, KAZ-S-11-2021, 12, KAZ-S-12-2021; 13, KAZ-N -1-2021; 14, KAZ-N -2-2021; 15, KAZ-N -3-2021; 16, KAZ-N -4-2021; 17, KAZ-N -5-2021; 18 KAZ-N -6-2021; 19, KAZ-N -7-2021; 20, KAZ-N -8-2021, 21, Greece 9 (positive control for *ToxB* gene); 22, ddH_2O (negative control for *ToxB* gene)

Table 3 – Results of genotyping wheat samples using the *Xfcp623* marker

Accessions	Host, cultivar	*Xfcp623*	
		bp	gene
Oral	*Triticum aestivum*	null	*tsn1*
KZ-KP32-2021	*Triticum aestivum*	380	*Tsn1*
KZ-KP33-2021	*Triticum aestivum*	null	*tsn1*
KZ-KP34-2021	*Triticum aestivum*	null	*tsn1*
KZ-KP35-2021	*Triticum aestivum*	380	*Tsn1*
KZ-KP36-2021	*Triticum aestivum*	null	*tsn1*
Akbiday	*Triticum aestivum*	380	*Tsn1*
Koksu	*Triticum aestivum*	null	*tsn1*
Alem	*Triticum aestivum*	380	*Tsn1*
Rosinka 3	*Triticum aestivum*	380	*Tsn1*
KZ-KP40-2021	*Triticum aestivum*	380	*Tsn1*
Aliya	*Triticum aestivum*	null	*tsn1*
KZ-KP44-2021	*Triticum aestivum*	null	*tsn1*
Reke	*Triticum aestivum*	null	*tsn1*
KSI16-2021	*Triticum aestivum*	null	*tsn1*
KSI17-2021	*Triticum aestivum*	null	*tsn1*
KZ-KP46-2021	*Triticum aestivum*	null	*tsn1*
Salamouni	*Triticum aestivum*	null	*tsn1*
Glenlea	*Triticum aestivum*	380	*Tsn1*
Note: *Xfcp623* – SSR marker of the *Tsn1* locus sensitive to Ptr ToxA amplifies a 380 bp DNA fragment			

As an example, the PCR results for 19 wheat samles are shown in the Figure 3. Seven entries (KZ-KP32-2021, KZ-KP35-2021, Akbiday, Alem, Rosinka 3, KZ-KP40-2021 and Glenlea) had 380 bp fragment, indicative of the dominant *Tsn1* allele conferring toxin Ptr ToxA sensitivity. Eleven entries (Oral, KZ-KP33-2021, KZ-KP34-2021, KZ-KP36-2021, Aliya, Koksu, KZ-KP44-2021, Reke, KSI16-2021, KSI17-2021 and Salamouni) had no amplification product (null allele), indicative of the recessive *tsn1* allele conferring toxin *Ptr* ToxA insensitivity.

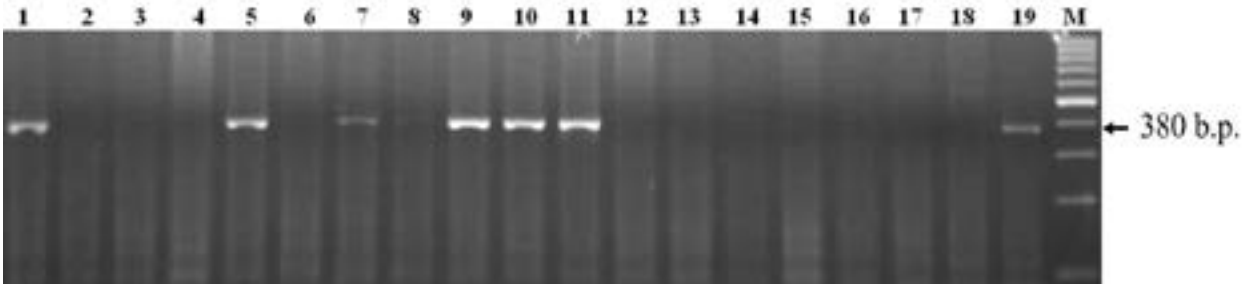

Figure 3 – DNA amplification product for wheat cultivars and elite lines obtained with diagnostic marker *Xfcp623* linked to the *Tsn1* gene sensitive to Ptr ToxA. Lane: 1, KZ-KP32-2021; 2, Oral; 3, KZ-KP33-2021; 4, KZ-KP34-2021; 5, KZ-KP35-2021; 6, KZ-KP36-2021; 7, Akbiday; 8, Koksu; 9, Alem; 10, Rosinka 3; 11, KZ-KP40-2021; 12, Aliya; 13, KZ-KP44-2021; 14, Reke; 15, KSI16-2021; 16, KSI17-2021; 17, KZ-KP46-2021; 18, Salamouni (resistant reference cultivar for race 1, insensitive to Ptr ToxA, with recessive gene *tsn1*); 19, Glenlea (susceptible reference cultivar for race 1, sensitive to Ptr ToxA, with dominant gene *Tsnl*); M, DNA ladder. Fragments amplified by *Xfcp623* were separated in 2% agarose gels. The bands are 380 bp for the *Tsn1* allele (lanes 1, 5, 7, 9, 10, 11 and 19), sensitive to Ptr ToxA and null allele for the *tsn1* allele, insensitive to Ptr ToxA (lanes 2, 3, 4, 6, 8, 12, 12, 13, 15, 16, 17 and 18)

Determination of the prevalence of genes encoding toxins in the local population, and the susceptibility of commonly grown wheat cultivars to Ptr aid selection of wheat cultivars to reduce disease risk. Thus, according to the results of our research, five races of *Ptr* (4, 5, 6, 7 and 8) have been identified from southern Kazakhstan, and three races (2, 4 and 7) from northern Kazakhstan. ToxB was not previously found in Kazakhstan, but the results of this study show the appearance and spread of this toxin in south Kazakhstan. According to the obtained data, the frequency of occurrence of isolates with the *ToxB* gene was 10.0%. A similar observations of occurrence frequency of ToxB was noted in studies by Kamel et al in 2019 in Tunisia [40]. In the present study, the wheat entries were genotyped with *Xfcp623* marker to predict reaction to the *Ptr* ToxA. Eleven wheat varieties resistant to HST ToxA were identified using molecular markers linked to the *tsn1* gene insensitive to *Ptr* ToxA.

Conclusion

Our results indicate an annual fluctuation in the population structure of *P. tritici-repentis* in the regions of Kazakhstan. Identification of six Ptr races on wheat demonstrates the high diversity of the pathogen population in Kazakhstan, which requires further in-depth annual studies. It was found that races 7 and 8 dominate in isolates from southern Kazakhstan, and race 4 prevails in northern Kazakhstan. Twenty single spore isolates of *P. tritici-repentis* were characterized for the presence of the *Ptr ToxA* and *Ptr ToxB* genes. Seven isolates were positive for the *ToxA* gene, and two isolates were positive for the *ToxB* gene. Eleven wheat varieties resistant to HST ToxA were identified using molecular markers linked to the *tsn1* gene, insensitive to Ptr ToxA. Our results have important practical implications for breeders when studying the distribution of *P. tritici-repentis*.

Acknowledgments

This study was conducted within the framework of the program OR11465424 “Development and implementation of highly effective diagnostic systems for identifying the most dangerous diseases and increasing the genetic potential of crop resistance”, task 01 “Monitoring the development of diseases to determine the level of danger of pathogens of brown, yellow rust and diseases of wheat leaf spots” for 2021-2022 supported by the Ministry of Science and

Higher Education of the Republic of Kazakhstan. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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