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Molecular identification and morphological characterisation of multiple fungal pathogens of triticale in Northern Kazakhstan

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Abstract

Background and Aim. Ttriticale is classified as an amphidiploids and is the first grain crop created by humans, possessing high yield potential along with favorable biochemical and technological characteristics. For a long time, it was believed that triticale, during selection, inherited disease resistance from wheat and resistance to abiotic factors from rye. However, in recent years, there have been several reports that triticale is to fungal diseases, which reduces the quality of the harvested crop.

The aim of our study is to examine and characterize the fungal pathogens of triticale in Northern Kazakhstan, and conduct molecular-genetic identification of the main fungal pathogens.

MaterialsandMethods. The study was conducted of triticale from two varieties, Dauren and Rossika. Primary fungal isolation was carried out on agarized nutrient media, with preliminary identification using microscopy. Molecular-genetic analysis was performed to determine the species of fungi.

Results. During the study, we isolated five major fungal pathogens from different parts of the plant. Data on the percentage of infection by the main fungal pathogens were provided. Three of them are pathogens of alternariosis – *Alternaria alternate* (more common in grains 39%, in leaves and scales of seeds 19-21%), fusariosis – *Fusarium tricinctum* (occurrence: in roots 57%, in grains 17%, in leaves 10%) and helminthosporiosis – *Bipolaris sorokiniana* (occurrence in roots and leaves 0.83%) of grain crops, which can lead to a decrease and loss of yield due to their production of mycotoxins. A cultural and morphological description of the main fungal pathogens of grain crops was provided. Molecular-genetic identification was carried out using the ribosomal marker ITS (internal transcribed spacer).

Conclusion. According to result of our research, we characterized and molecular-genetically identified the most common fungi found on different parts of the triticale plant.

Keywords: triticale; fungal pathogens; aternariosis; fusariosis; helminthosporiosis; molecular-genetic identification.

Introduction

As the global population continues to grow each day, the demand for grain-derived products is also increasing. Agriculture plays a crucial role in the economic, social, and environmental development of Kazakhstan, and is also the largest grain producer in Central Asia [1]. Triticale selection has been carried out in Kazakhstan since 1970.

Currently, triticale selection is aimed at sampling high-yield varieties with resistance to fungal diseases [2]. However, crop yields are influenced by environmental conditions, climate change, herbivorous insects that damage plants, as well as fungal and viral diseases. In triticale cultivation, the prevalence of diseases caused by various pathogenic fungi is significant, and factors such as pathogen type, weather conditions, and humidity affect disease incidence [3].

Fungal diseases are an important factor limiting the yield of grain crops and have been identified in virtually all countries and regions of the world. Economically important diseases of cereals include diseases caused by the pathogens *Blumeria graminis, Puccina recondita, Puccinia graminis, Puccina striiformis, Septoria tritici, Septoria nodorum,* and *Fusarium* [4]. Common pathogens of triticale include species such as *Bipolaris sorokiniana, Alternaria, Fusarium, Rhizopus*, and *Penicillium.* These fungi are the main causative agents of diseases such as root rot and black rot. One of the key factors significantly influencing the occurrence of fungal diseases is weather conditions that are beyond human control. Fungal diseases contribute both to reduced crop yields and to the deterioration in crop quality [5].

In Northern Kazakhstan, as well as in the western and eastern regions, the most common diseases are helminthosporous - fusarium root rot, leaf rust, stem rust, septoria leaf spot and yellow wheat spots. In recent years, fungal diseases of grain crops have appeared in many countries of the world and are considered one of the main factors affecting yield and quality of agricultural crops. Throughout the entire growing season, grain plants are affected by many pathogens. Limited crop rotation is considered to be the main cause of fungal diseases [6]. This situation necessitates protective measures throughout the growing season [7].

The most common method of controlling pathogens of grain crop are fungicides. However, it has been noted that certain species develop resistance to the active ingredients contained in plant protection products [8]. The use of chemical plant protection is also associated with environmental pollution due to the residual presence of active substances in soil and grain [9]. Therefore, the search is underway for alternative biological methods to combat pathogens by fungi. A relatively large number of fungal-resistant plant forms can be found within the biological diversity of wild wheat species and crops such as spring or winter triticale.

Triticale (×Triticosecale Wittmack) is a synthetic hybrid obtained by crossing wheat (*Triticum sp*.) and rye (*Secale sp.*). The combination of qualities such as high productivity inherited from wheat and resistance to environmental factors acquired from rye in one hybrid allowed triticale to gain worldwide recognition [10]. Triticale combines the high yield potential and good grain quality of wheat with resistance to fungal diseases, including powdery mildew, leaf rust, yellow rust, and stem rust [11].

The aim of our work is to characterize and perform genetic identification of various fungal pathogens of triticale in Northern Kazakhstan, including *Bipolaris sorokiniana, Alternaria, Fusarium, Rhizopus,* and various species of *Penicillium.*

Materials and Methods

The study was conducted on triticale samples grown in the North Kazakhstan and Akmola regions, including 15 samples of the Dauren variety and 15 samples of the Rossika variety.

The seed material fully complied with the requirements of GOST 12044-93, "Seeds of Agricultural Crops: Methods for Determining Disease Contamination".

The seeds of hybrid plant forms, spring soft wheat varieties, and spring triticale were first washed under running water for 1-2 hours. They were then disinfected with 96% alcohol for 1-2 minutes. After disinfection, the seeds were rinsed with sterile water and dried between layers of sterile filter paper. Ten seeds were placed in each Petri dish and incubated in a thermostat at 25-27 °C for germination, a process that typically lasted from 7 to 10 days. To examine the seeds for the presence of pathogens, a small portion of the growing colony was observed in a drop of water under a Zeiss AxioScope A1 microscope.

Genomic DNA was extracted from fungal strain using liquid nitrogen and phenol-chloroform extraction method, and the genomic DNA was analyzed by electrophoresis on 1% agarose gel. The ITS

region on rDNA was amplified by using specific primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (Integrated DNA Technologies, Inc., USA). The PCR reaction was done in a SimpliAmp thermal cycler (Applied biosystems) under the following conditions: an initial denaturation set up at 94 °C for 5 min was followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 52 °C for 40 sec and extension at 72 °C for 50 sec, with a final extension step of 72 °C for 7 min.

PCR samples were purified from oligonucleotide residues by dephosphorylation using alkaline phosphatase (SAP - shrimp alkaline phosphatase) and endonuclease. A mixture was prepared in a total volume of 10 µl for each sample - dH2O - 7.25 µL, 10× PCR Buffer - 1.0 µl, MgCl2 - 1.0 µl, SAP (5 mM) - 2.5 µl, Exonuclease I (5 units/µL) - 0.125 µl. The resulting mixture was added to each PCR product, placed in a thermal cycler under the following conditions: 37 °C - 30 min, 85 °C - 15 min, 4 °C - ∞. Sample preparation for sequencing carried out by precipitation with an alcohol-acetate mixture.

The components of a standard set of reagents for the sequencing reaction were prepared in a 0.2-ml thin-walled thermocycler tube. A standard set of reagents for cyclic sequencing using CEQ WellRED terminator dyes (partially mixed). The following thermal cycle program was chosen: 96 ℃ - 20 sec, 50 ℃ - 20 sec, 60 ℃ - 4 min for 30 cycles and followed by aging at 4 ℃. The sequencing was done by using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and the sequence was deposited in GenBank. These sequences were compared with other sequences in the GenBank by using the BLAST analysis.

Results

As part of the study to identify phytopathogenic fungi on triticale, the entire plant was examined, from the root to the spike (Figure 1).

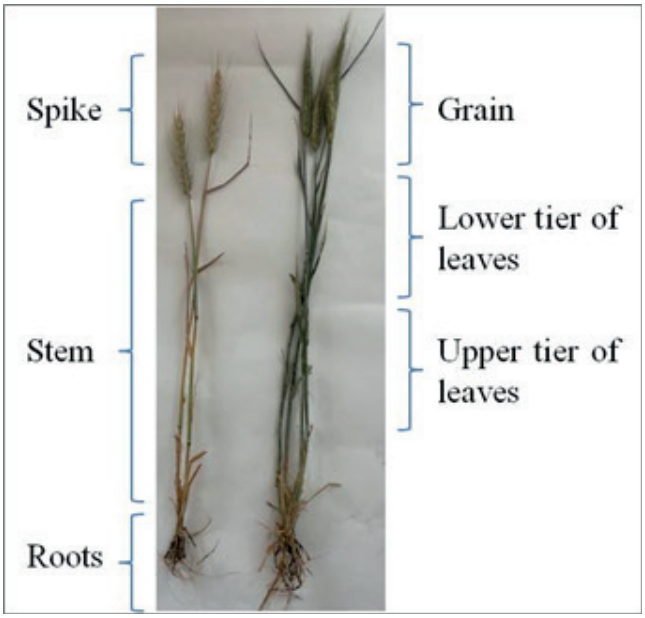
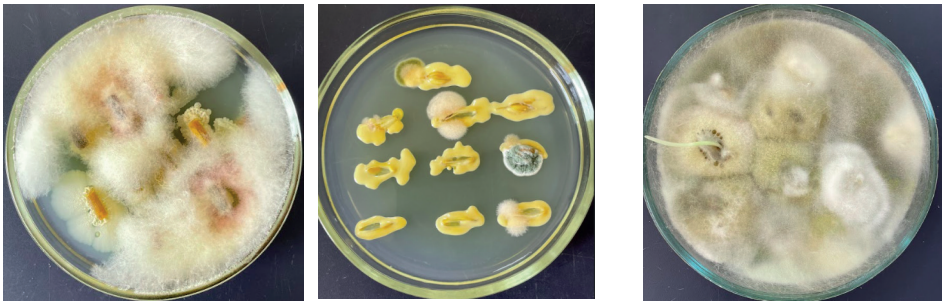


Figure 1 – Triticale with highlighted study areas

In Figure 1, the scheme shows the triticale areas selected for investigation to identify phytopathogenic fungi.

After separating the selected areas, the samples were sterilized according to GOST 12044-93, "Seeds of Agricultural Crops: Methods for Determining Disease Contamination". The cultures were sown on Potato-Glucose Agar medium. Incubation was conducted at 25-27 °C in an incubator for 5-10 days. Images of the primary sowing of triticale parts are presented in Figure 2.

roots lower tier of leaves upper tierofleaves



stem spike grain

Figure 2 – Primary sowing by sites of whole triticale plant

As shown in Figure 2, a variety of phytopathogenic fungi grew after the primary sowing.

Based on the initial phytosanitary analysis, we compared the percentage of pathogenic fungi detected in the Dauren and Rossika varieties.In Rossika roots, the occurrence of Fusarium spp. was 12.8%, while in Dauren it was 12%, and Bipolaris spp. was 1%. Leaves were affected by Alternaria spp.: with 11% in Dauren and 9.2% in Rossika. The occurrence of Alternaria spp. in grains was the same for both varieties at 12%, while Fusarium spp. occurred at 10.71% in Rossika and 5% in Dauren. Saprophytic fungi were found in Rossika at 8.5%, and in Dauren at 1%.

Based on this comparison, it can be concluded that the Dauren variety is more resistant to fungal diseases of grain crops than the Rossika variety.

We also conducted a statistical analysis of the percentage of major fungal infections detected in different parts of the plants (Figure 3).

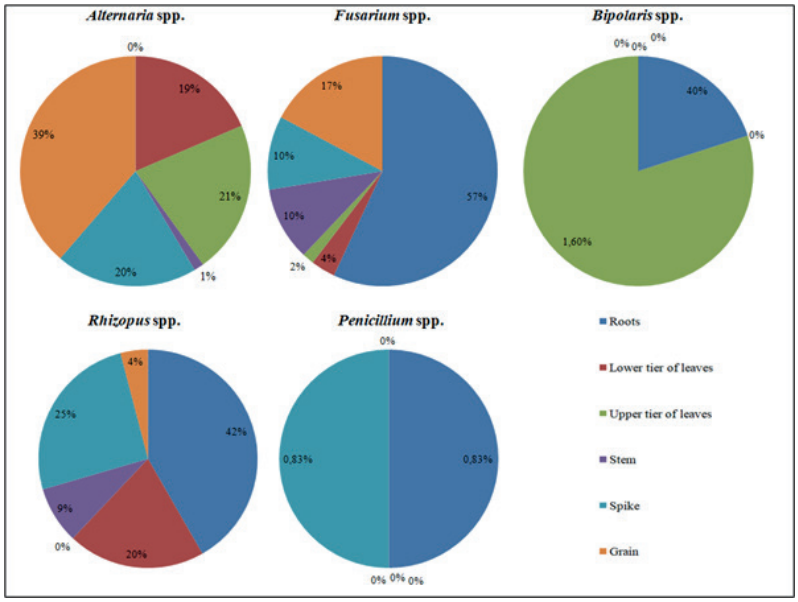
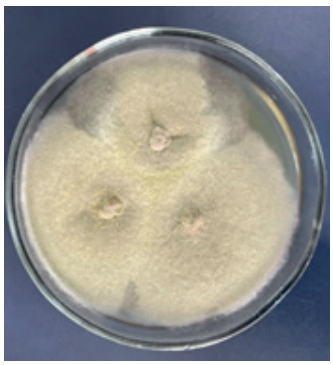
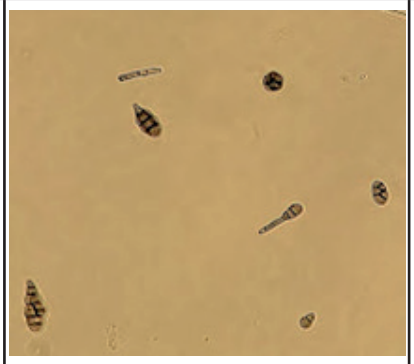
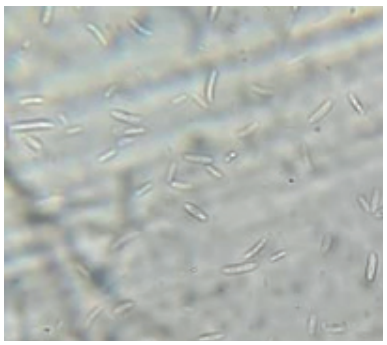
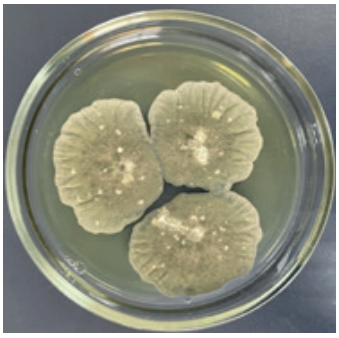
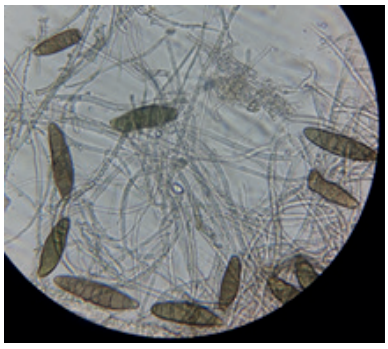
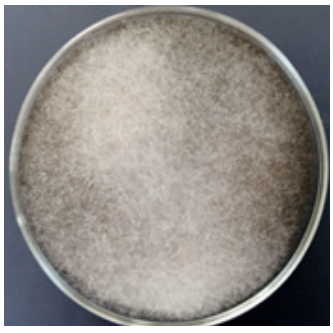
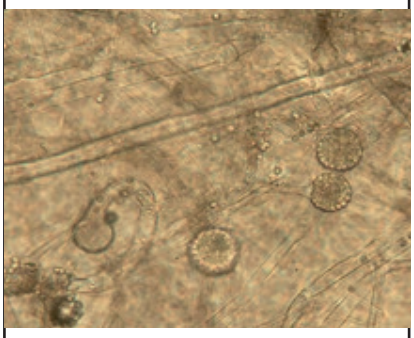


Figure 3 – Diagrams on the percentage of fungal infections

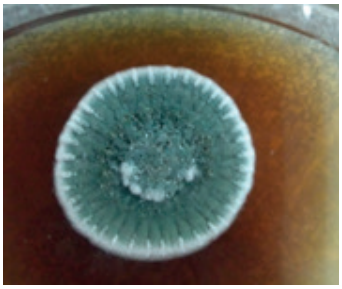
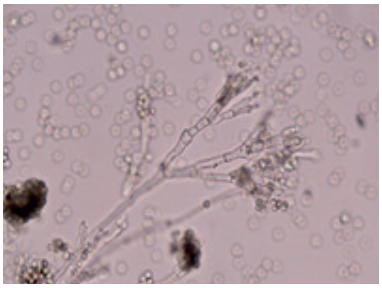
Figure 3 presents pie charts showing the percentage of infection by major fungal pathogens. For example, *Alternaria spp.* predominantly affects the grains at 39%, while it is less common in the lower and upper tiers of leaves and on the spike, at 19-21%, accordingly it is practically not found in the roots and stems of the plant. *Fusarium spp.* is most commonly found in the roots of the plant at 57%, less frequently in the grains at 17%, and in the spike and stems at 10% each. *Bipolaris spp.,* the causative agent of helminthosporiosis, is most found in the roots and upper tiers of leaves. *Rhizopus spp.* and *Penicillium spp.,* being saprophytic pathogens, can be localized on all parts of the plant.

The prevailing isolates reseeding to isolate pure cultures (Table 1).

Table 1 – Pure cultures of the main pathogens of triticale phytopathogens and their microscopy

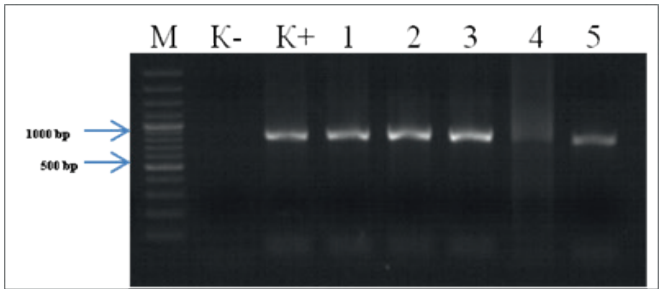
Pure culture	Microscopy	Primary identification
		*Alternaria spp.* – colony with straight edges,a velvety flake-like surface of gray-olive color, moderate growth rate. The hyphae are septic, from olive to dark brown in color. Conidia are club-shaped, pear-shaped, ovoid with a short conical spout, with 6-12 transverse and 1-5 longitudinal partitions, smooth-walled or warty.
		*Fusarium spp.* – colony with straight edges, with a cotton-wool air mycelium, the surface is whitish to yellowmoderate growth rate. The hyphae are septic, colorless.The macroconidia are sickle-shaped, with 3-5 partitions, slightly curved. Microconidia and blastoconidia are fusiform, with 2-4 partitions.
		*Bipolaris spp.* – the colony has rough edges, velvety and woolly, the surface is gray to olive green in color, the colony is wrinkled, the growth rate is moderate. The hyphae are septic, brown. Conidia are club-shaped, slightly curved with 2-14 (usually more than 6) transverse partitions, germinate bipolar.
		*Rhizopus spp*. – colonies with straight edges, the surface is loose, with a tight cotton coating, from white to brownish-gray in color, the growth rate is moderate, The rhizoids are branched, dark brown. 2-4, rarely 5 sporangiophores with sporangia depart from the neck of the rhizoid. Sporangiophores are formed on mycelium hyphae. Each forms from 2 to 11 sporangia. Sporangia are colorless, spherical. They are light brown, striated up to 5-9 microns long.

Continuation of Table 1

		*Penicillium spp.*–the colony has straight edges, the surface is velvety green, the edges are lighter, the colony is wrinkled, the growth rate is moderate. Hyphae are colorless, monopodial branched, with partitions.Conidia are spherical, elliptical, smooth-walled or warty.

According to Table 1, there are presented frequently encountered phytopathogens of grain crops and their microscopy, which makes it possible to identify isolates before generic affiliation.

Molecular genetic analysis allows to identify isolated phytopathogenic fungi to the species. The results of the electrophoregram are shown in Figure 4.



M – DNA ladder (100 bp); K- – negativecontrol; K+ – positivecontrol; 1-5 – DNA

Figure 4 – Electrophoregram results of phytopathogenic fungies

The obtained PCR products were sequenced by Sanger. The obtained nucleotide sequences were tested on the platform of the NCBI international database: 1 – *Alternaria alternate,* 2 – F*usarium tricinctum*, 3 – *Bipolaris sorokiniana*, 4 – *Rhizopus arrhizus*, 5 – *Penicillium chrysogenum*.

A bioinformatic analysis was performed with the obtained nucleotide sequences with the construction of a phylogenetic tree (Figure 5).

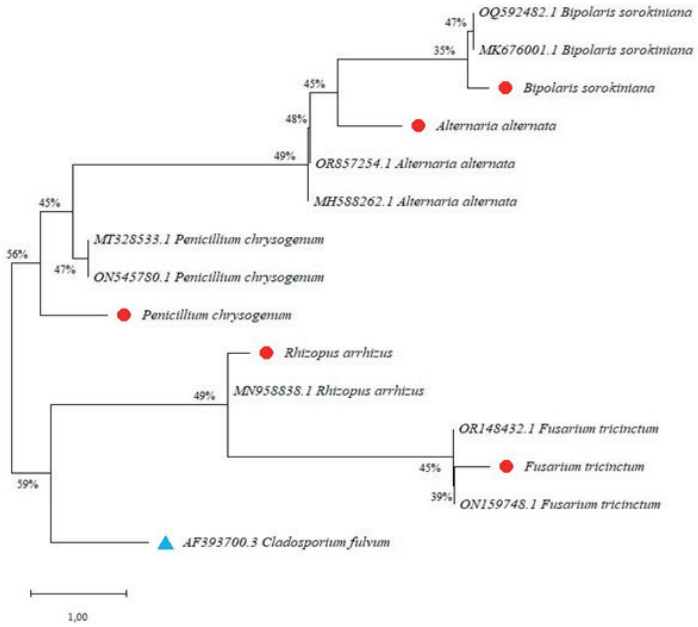


Figure – 5 Phylogenetic tree of nucleotide sequences of the obtained pathogenic triticale fungies

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model [12]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1144 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [13].

Thus, we examined triticale samples for phytopathogenic fungi. Frequently occurring fungi in triticale have been characterized and genetically identified up to the species composition, and bioinformatic analysis has been carried out with the construction of a phylogenetic tree.

Discussion and Conclusion

Fungal diseases affecting grain crops, including triticale, can attack plants during the growing season, harvesting, as well as in case of violation of the seed storage regime. In our study were found *Bipolaris sorokiniana, Alternaria, Fusarium, Rhizopus* and *Penicillium.* These fungi are the main causative agents of diseases such as root rot and black rot.

As a result of our studies between samples of Dauren and Rossika varieties, we compared the percentage of detection of pathogenic fungi in two varieties. In the Rossika variety, the occurrence of phytopathogenic fungi in the roots of *Fusarium spp*. 12.8%, in the Dauren variety *Fusarium spp.* 12%, *Bipolaris spp.* 1%. The leaves are exposed to *Alternaria spp*.: Dauren 11%, Rossika 9.2%. Occurrence in grains of *Alternaria spp*. the two varieties have the same 12%, *Fusarium spp.* The Rossika have 10.71%, Dauren 5%. Saprophytic fungi affect the plant in the Rossika 8.5%, and in the Dauren 1%.

Studies in India and Brazil have shown that fungal diseases of grain cultures are usually favored by warm weather [14]. In addition, high humidity is an important factor in increasing the development of symptoms [15].

It is well known that fungal diseases contribute to both a decrease in yield and a deterioration in crop quality.

In the research of scientists Motzo et al. [16] emphasised that the yield of the variety depends on environmental conditions at various phases of the growing season of the variety, which is consistent with our research.

According to the results of our research, it was revealed that the Dauren variety is more resistant to fungal diseases of grain cultures than the Rossika variety.

As a result of the study, pathogenic fungi were identified that are pathogens of such grain diseases as alternariosis – *Alternaria alternate,* fusariosis – *Fusarium tricinctum* and helminthosporiosis – *Bipolaris sorokiniana*. Fungal diseases lead to high yield losses because they reduce the assimilating area of leaves and spikes, which leads to poor grain formation and a decrease the number of grains in the spikes. Mycotoxins are secondary fungal metabolites with low molecular weight produced by fungi of the genera: *Aspergillus, Penicillium, Fusarium* and *Alternaria,* which can potentially produce various mycotoxins in the field or during storage of cereals due to poor storage conditions. It is recommended to conduct additional research to reduce the dangerous effects on animal and human health,caused by pollution with fungal pathogens. Currently, fungicides are the most common method of combating pathogens of fungal diseases in grain cultures.

Authors' Contributions

AS, UI, VS, VK: Concept development, design and planning of the study, data collection and analysis, critical review of the article and final approval, research, statistical analysis. OY and LC: Conducted the final revision and proof reading of the manuscript. VS and VV: Conducted a comprehensive literature search and conducting research. All the authors have read, reviewed and approved the final version of the manuscript.

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Солтүстік Қазақстандағы тритикале саңырауқұлақ патогендерінің әртүрлі түрлерінің сипаттамасы және генетикалық идентификациясы

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Түйін

Алғышарттар мен мақсат. Тритикале амфидиплоидтарға жатады және қолайлы биохимиялық және технологиялық сипаттамалары бар жоғары өнімділікке ие адам жасаған алғашқы дәнді дақыл. Ұзақ уақыт бойы тритикале селекция барысында бидайдан дәнді дақылдардың әртүрлі ауруларына, ал қара бидайдан абиотикалық факторларға төзімділікке ие болды деп есептелді. Соңғы жылдары тритикаленің саңырауқұлақ ауруларына бейімділігі нәтижесінде алынған өнімнің сапасын төмендететіні туралы бірқатар мәліметтер бар.

Біздің жұмысымыздың мақсаты Солтүстік Қазақстандағы тритикале саңырауқұлақ патогендерінің қоздырғыштарын зерттеу және сипаттау, сондай-ақ саңырауқұлақ патогендерінің негізгі қоздырғыштарын молекулалық-генетикалық идентификациялау болып табылады.

Материалдар мен әдістер. Зерттеу Даурен және Россика екі сортының тритикале үлгісінде жүргізілді. Саңырауқұлақтарды бірінші рет бөліп алу микроскопия көмегімен алдын ала идентификациялау арқылы агар қоректік орталарында жүргізілді. Саңырауқұлақтардың түр сәйкестігін анықтау үшін молекулалық-генетикалық талдау жүргізілді.

Нәтижелер. Зерттеу барысында біз өсімдіктің әртүрлі бөліктерінен бес негізгі саңырауқұлақ қоздырғыштарын анықтадық. Мәліметтер негізгі саңырауқұлақ инфекцияларынан туындаған

зиянның пайызы туралы берілген. Оның үшеуі – *Alternaria – Alternaria alternate* (көбінесе дәнде 39%, жапырақтарда және тұқым қабыршақтарында 19-21%), *Fusarium – Fusarium tricinctum* (кездесу: тамырда 57%, дәнде 17%, жапырақта 10) қоздырғыштары %) және гельминтоспориоз – *Bipolaris sorokiniana* (тамыр мен жапырақта 0,83%) дәнді дақылдарда кездеседі, бұл микотоксиндердің өндірілуіне байланысты өнімділіктің төмендеуіне және жоғалуына әкелуі мүмкін. Дәнді дақылдардың саңырауқұлақ ауруларының негізгі қоздырғыштарына культуралды-морфологиялық сипаттама берілген. ITS (internal transcribed spacer) рибосомалық маркер аймағы бойынша молекулалық-генетикалық идентификация жүргізілді.

Қорытынды. Жүргізілген зерттеулердің нәтижесінде біз барлық тритикале өсімдігінің әртүрлі аймақтарында жиі кездесетін саңырауқұлақтарды сипаттадық және молекулалық-генетикалық түрде анықтадық.

Кілт сөздер: тритикале; саңырауқұлақ патогендері; альтернариоз; фузариоз; гельминтоспориоз; молекулалық-генетикалық идентификация.

Характеристика и генетическая идентификация различных видов грибных патогенов тритикале Северного Казахстана

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Аннотация

Предпосылки и цель. Тритикале относится к амфидиплоидам и является первой зерновой культурой, созданной человеком, которая обладает высоким потенциалом урожайности с благоприятными биохимическими и технологическими характеристиками. Долгое время считалось, что тритикале в ходе селекции получила от пшеницы устойчивость к различным болезням зерновых культур, а от ржи – устойчивость к абиотическим факторам. В последние годы, имеется ряд сообщений о том, что тритикале подвергается грибным болезням, что снижает качество полученной продукции.

Целью нашей работы является изучить и дать характеристику возбудителям грибных патогенов тритикале Северного Казахстана, а также провести молекулярно-генетическую идентификацию основных возбудителей грибных патогенов.

Материалы и методы. Исследования проводили на образцах тритикале по двум сортам Даурен и Россика. Первичное выделение грибов проводили на агаризованных питательных средах с предварительной идентификацией, с помощью микроскопирования. Для установления видовой принадлежности грибов проводили молекулярно-генетический анализ.

Результаты. В ходе исследования нами было выделено пять основных грибных патогенов с разных участков растения. Приведены данные по проценту поражения основными грибными инфекциями. Три из них являются возбудителями альтернариоза - *Alternaria alternate* (чаще встречается в зернах 39%, в листьях и чешуйках семян 19-21%), фузариоза – *Fusarium tricinctum* (встречаемость: корни 57%, в зерне 17%, в листьях 10%) и гельминтоспориоз – *Bipolaris sorokiniana* (встречаемость в корнях и листьях 0,83%) зерновых культур, которые могут привести к снижению и потери урожайности за счет продуцирования ими микотоксинов. Дана культурально-морфологическая характеристика основных возбудителей грибных заболеваний злаковых культур. Проведена молекулярно-генетическая идентификация по рибосомальному маркерному участку ITS (internal transcribed spacer).

Заключение. В результате проведенных исследований, нами охарактеризованы и молекулярно-генетически идентифицированы наиболее часто встречаемые грибы на разных участках целого растения тритикале.

Ключевые слова: тритикале; грибные патогены; альтернариоз; фузариоз; гельминтоспориз; молекулярно-генетическая идентификация.