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MOLECULAR-GENETIC IDENTIFICATION OF CAUSAL AGENTS OF FUNGAL DISEASES ON POTATO AND TOMATO COLLECTED UNDER PRODUCTION STORAGE

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ABSTRACT

The expansion of exports and imports of potato and tomato crops between countries contributes to the rapid spread of the most harmful species and strains of phytopathogenic fungi around the world. Due to it, the identification of phytopathogenic fungi is important for agriculture. Currently, molecular-genetic methods are considered as one of the modern and promising methods for the diagnosis and identification of plant diseases, which allow identifying microorganisms without any knowledge of its biological features. One of the recommended methods for identification and classification of taxa is the determination of the nucleotide sequence of nuclear ribosomal gene regions.

276 infected potato tubers and tomato fruits were used to identify phytopathogenic fungi isolates, which were collected from production storages. Molecular-genetic identification of microorganisms based on the analysis of the nucleotide sequences of the ITS region made it possible to taxonomically identify 102 isolates of phytopathogenic fungi that belong to 7 genera. It was revealed that the proportion of fungi, which belohg to the genera *Fusarium*(60%), *Penicillium* (15%) and *Clonostachys* (12%), *Alternaria* (9%) were dominated in the generic structure.

The Isolation frequency *(Fr)* and relative density *(RD)* of 7 generaphytopathogenic fungi were calculated according to the results of molecular-genetic identification.Isolation frequency *(Fr)* of fungi genera ranged from 0.3% to 22.1%. Relative density of fungi species ranged varied 0.9% to 59.8%.

Thereby, molecular-genetic identification based on the analysis of the nucleotide sequence of the ITS region made it possible to perform identification of pathogens on potato and tomato under production storage. Also, fungi *Clonostachys* and *Acrostalagmus* genera were isolated, which are not pathogens of potato and tomato diseases.

Key words: potato, tomato, fungi, ITS region, identification, isolation frequency, isolation relative density.

INTRODUCTION

Kazakhstan is located in the Central Asia. It has a population of 17.54 million inhabitants (6 people per sq. km of land area) [1].

Russia, China and the nearby countriesare the main trade partners of Kazakhstan [2]. Although, Kazakhstan has large territory of fertile land. However, the country has environmental problems such as water scarcity, harsh climate conditions and fungal diseases.

Potato and tomato are universal highly popular over the world [3]. In many countries and in urban areas, level of its consumptionarerising. Because of that, demand for potato and tomato is increasing. The government of Kazakhstan givesmore privaty to support the well-being of its population [4]. Due to it,

Kazakhstan has national development programmes, which force to support development of agriculture and food industry, especially for cultivation and promotion of cereals, fruits and vegetables [5- 7]. Growing potatoes is very important for Kazakhstan, as it is one of the main food products for population.The average planted area under this crop in the republic is about 186.3 thousand ha, however, due to the low yield, the gross harvest of tubers does not meet the needs of agriculture. From 2015 to 2017, potato production in Kazakhstan increased marginally from 3.521.0 to 3.551.1 thousand tons and yields ranged from 185.5 centner/ha to 194.2 centner/ha [8].

Among vegetable crops, tomato is one of the most important year-round sources of vitamins, organic acids, pectins and valuable minerals.Tomatois considered as an essential source of antioxidants ćly - copenecandecrease risk of chronic diseases, such as cancer and cardiovascular disease [9]. Tomato yield does not fully meet the needs of the population.One of the reasons for the low tomato yield is the defeat of a wide range of diseases - fungal, bacterial and viral etiology [10]. According to Kazenas L.D. 28 diseases of tomatoes are registered in Kazakhstan [11].

The planted area for tomatoes was 29.485 ha, the gross harvest of tomato fruits - 739 tons and yield - 250.638 hectagram per hectar in Kazakhstan in 2017 [12].

To meet the needs of the Kazakhstan population, potatoes are imported annually from countries near and far abroad, such as Iran, Pakistan, Russia, Belarus, Germany, China, Netherlands, Georgia, Azerbaijan, Tajikistan, Afghanistan, and Kyrgyzstan [13].

The expansion of export and import between countries contributes to the rapid spread of the most harmful phytopathogenic fungi species and strains around the world.

Late blight is a common disease of plants such as tomatoes and potatoes, capable of wiping out entire crops on commercial-scale fields. Caused by a fungus-like pathogen, it first appears as black or brown lesions on leaves, stems, fruit or tubers. Today, late blight still causes more than 6.7 billion dollars in annual losses worldwide. [14], therefore, the identification of phytopathogenic fungi is important for agriculture. Constant and accurate control over the phytosanitary state of the environment and agricultural plants ensures food and biological safety.

For disease diagnosis is used microscopy of affected tissues to detect a pathogenic microorganism and its identification by the structure of mycelium and sporulation characteristics [15]. Microscopic analysis is the most common in the practice of specialized laboratories for agriculture.Disadvantages of microscopic analysis is that many fungi are difficult to cultivate or unculturable [16].

Direct detectionof fungi enables a deeper understanding of natural microbial communities, which can beperformed quickly and accurately [17]. One of the recommended methods for identification and taxonomic classification of phytopathogens is the determination of the nucleotide sequence of nuclear ribosomal gene regions [17]. Pryce et al. in their work showed that ITS regions can be used to identify fungi [18]. Conrad L. and colleagues have shown that the internal transcribed spacer (ITS) region has the highest successful probability of identification for a wide range of fungi [19].

The aim of this work is to molecular-genetic identification of causal agents of fungal diseases on potato and tomato collected under production storage.

Materials and methods

Collection of infected potato tubers and tomato fruits. Infected potato tubers and tomato fruits was collected in 2018 (spring) from storage of RSE flNational Center for BiotechnologyScience Comtee Ministry of Education and Science of the Republic of Kazakhstan (Stepnogorsk) and market (Shanghai, Nur-Sultan). The total number of samples made up 276 potato tubers and tomato fruits.

Isolation and pure culture of fungi. In order to isolate pathogenic fungi from potato tubers and tomato fruits, studied plant materials were preliminary washed from soil and various impurities. The surface of the plant materials was sterilized in 1% sodium hypochlorite for 2 minutes. Healthy potato tubers and tomato fruits were sterilized with 75% ethanol and used as a control. A sterile small piece was cut off at the border of infected and healthy tissue and placed on an agar medium PDA (Potato infusion 200 gm, dextrose 20 gm, agar 20 gm, DW) and incubated in a thermostat at 28° C for 2 weeks.

After isolation of cultures from infected potato tubers and tomato fruits, they were purified from other organisms and microorganisms are maintained in a viable state free of contamination. The isolated pure cultures were subcultured on Czapek agar medium.

DNA extraction. DNA was extracted from two-day cultures of phytopathogenic fungi isolated from infected potato tubers and tomato fruits using the

[20] phenol-chloroform method

Direct DNA sequence determination. For genotyping of the ITS region, a polymerase chain reaction (PCR) was performed using primers: ITS4 (8243) ć tcctccgttattgatgc и ITS5 ć ggaagtaaaagtcgtaacaagg (Sigma Aldrich, UK).

Amplification was performed according to the following conditions (table 1).

Table 1. ITS region amplification conditions

PCR mix		Parametrs	
Reagents	Volume	PCR cycling parameters	Cycles
Forward ITS4 (5 pmol)	0.8µl	94°C - 5″	-
Reverse ITS5 (5 pmol)	0.8µl	95°C - 30′	30
10xBuffer	3 µl	52°C - 30′	
dNTPs (0.2 mM)	3µl	72°C ć 50′	
MgCl 2mM	3µl	72°C ć 7″	-
mQ	Up to 20 µl	Storage 4°C	-
Taq Polymerase	1 u	-	-
DNA(50 ng)	5 µl	-	-

Subsequently, electrophoresis was performed on 2% agarose gel with 5 µl of the reaction mixture. The results were documented using a gel recording system «BIO-RAD».

The dephosphorylation of the 5≥-terminal phosphate groups in the amplified reaction mixture was carried out during incubation with 0.5 unit alkaline phosphatase of Arctic shrimp (Shrimp Alkaline Phosphatase, Fermentas) minutes at 37°C for 30 andfollowed by inactivation of the enzyme by heating for 10 minutes at 85°C. Direct DNA sequence determinationwas performed according to the Sanger method using the BigDye Terminatorv 3.1 Cycle sequencing Kit (table 2).

Table 2. Sequence PCR parametrs

Mix for PCR		Parametrs	
Reagents	Volume	PCR cyclingparameters	Cycles
Forward/Reverse (3.2pmol)	1 µl	96°C ć 1′	-
5xBuffer	1 µl	96°C ć 10″	25
Terminator Ready Reaction Mix	1 µl	50°C ć 5″	
mQ	Upto 10 µl	60°C ć 10′	
PCR product	2µl	Storage 4°C	-

Purification of the reaction mixture from unbound components. Purification of the reaction mixture from unbound components was carried out with the mixed solvents composed of ethyl-acetate.

Ethyl-acetate (40 µl):

3M CH_3COONa (pH 4.6-5.2) ć 1.5 µl;

C_2H_5OH (95%) ć 31.3 µl;

dH_2O ć 7.25 µl.

40 µl of ethyl-acetate mixture was added to each tube. It was held at room temperature for 30 minutes, and then centrifuged at 12000 rpm for 30 minutes. The supernatant was removed, 80 µl C2H5OH (75%) was added to the precipitate, then centrifuged at 12000 rpm for 30 minutes. The supernatant was removed, the precipitate was dried at room temperature for 15 minutes. Then, 14 µl of Hi-Di Formamid (Applied Biosystems) was added to precipitate. Then, products maintained for 20 minutes at room temperature and denatured at 95°C for 2 minutes; (cooled in ice for 5 minutes). Direct sequence determination of ITS region was used for identification of species with followining determination of the nucleotide identity with the sequences deposited in the international GenBank data base (http://www.ncbi.nlm.nih.gov/genbank/).

Isolation frequency *(Fr) and*relative density *(RD)* and of fungi based on molecular identification (genera and species).

Relative density*(RD)* and isolation frequency *(Fr)* of genus were determined according to as follows: [21, 22, 23].

Isolation frequency *(Fr)* of fungi species was calculated according to formula 1:

$$Fr\,(\%) = \frac{(ns)}{N} \times 100 \qquad (1)$$

Where, ns is the number of samples on which a fungus occurred; N is the total number of seeds sampled.

The relative density *(RD)* of fungi species was calculated according to formula 2:

$$RD\ (\%)=\frac{(ni)}{N}\times 100 \qquad (2)$$

Where, ni is the number of isolates of a fungal genus, and N is the total number of fungal isolates obtained.

RESULTS AND DISCUSSION

One hundred and one mould isolates (in 7 genera) were obtained from 189 infected potato tubers and 87 tomato fruits (figure 1).

A B C

(A) potato tubers in production storage; (B) infected potato tuber; (C) selected tomato fruits;

Fig.1. Infected potato and tomato plants selected at production storages

For molecular-genetic identification of micro organisms, 102 pure cultures were obtained from collected plant materials (figure 2).

A B C

(A) *Clonostachys;* (B) *Acrostalagmus;* (C) *Fusarium*

Fig. 2. Pure cultures of Fungi

Molecular-genetic identificationusing the analysis of the nucleotide sequences of the ITS region allowed to provide taxonomic classificationof 102 isolates of phytopathogenic fungi. Occurrences of representatives *Fusarium* and *Penicillium* genera prevailed others with account of 60% (61 out of 102 samples) and (15%) (16 out of 102 samples), respectively (figure 3). This is relevant to the findings of [24] who identified *Fusarium, Penicillium, Butryodiplodia and Rhizopus* species from potato cultivars. This study has shown that fungal rot is the greatest cause of spoilt of potato tubers in storage. Colonization of the tubers by fungi will lead to reduction in consumption materials and itsvalue. Moreover, Kazerooni et.al revealed that is *Aspergillus* and *Fusarium species* are most abundant class in tomato [25].

Occurrences of *Clonostachys, Alternaria, Acrostalagmus, Cladosporium*, and *Nectria*genera was different (12%, 9%, 2%, 1%, and 1%, respectively) (figure 3).

In addition to potato pathogens, fungi of *Clonostachys* and *Acrostalagmus* genera were isolated. These fungi are not causal agents of potato diseases, since they are contained in the soil mycobiota [26]. During potato storage, there are soil residues, which can consist various specious of fungi that are not phytopathogenic microorganisms. Many of them able to winter in stored tubersOwing to the fact that potato tubers are rich in water and starch, they can be considered asa favorable substrate for the development of other microscopic fungi.

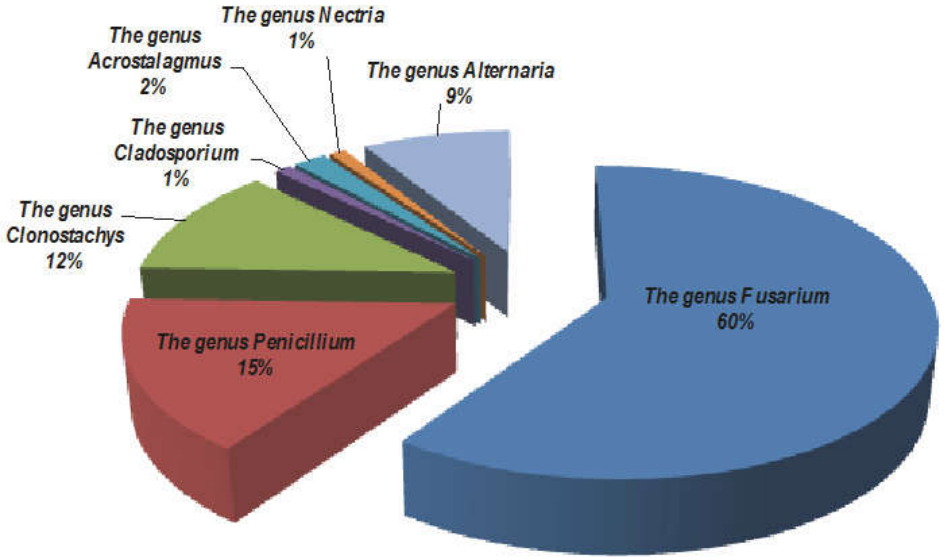

Fig.3. Identified fungal isolates (n=102)

Using ITS region sequences, Fiers et al. studied diversity of microorganisms associated with atypical superficial blemishes of potato tubers in France. They reported that the most represented fungi belonged to the genera *Fusarium, Rhizoctonia, Alternaria, Penicillium and Clonostachys*[27]. Emil Stefańczyk et al. studied the diversity of Fusarium spp., which are associated with dry rot of potato tubers in Poland. In our work, about 60% of samples belonged to the genus *Fusarium* [28].

Isolation frequency *(Fr)* and relative density (*RD*) of 7 fungi genera were calculated with following formula 1 and 2 for obtained. Calculation of isolationfrequency *(Fr)* and isolation relative density (*RD*) of fungi based on molecular identification.

In our study seven genera were recovered. Isolation frequency *(Fr)* and isolationrelative density (*RD*) are showed in Table 3. Among the studied samples, the isolation frequency (*Fr)* of fungi genenera ranged from 0.3% to 22.1%. Isolation relative density (*RD*) of fungi ranged from 0.9% to 59.8% (table 3).

Table 4.Fungal genera present in samples

№ №	Fungi	Number of isolates	*Fr (%)*	*RD (%)*
1	*The genus Fusarium*	61	22.1	59.8
2	*The genus Penicillium*	16	5.7	15.6
3	*The genus Clonostachys*	12	4.3	11.7
3	*The genus Cladosporium*	1	0.3	0.9
5	*The genus Acrostalagmus*	2	0.7	1.9
6	*The genus Nectria*	1	0.3	0.9
7	*The genus Alternaria*	9	3.2	8.8
Note: *Fr fk Isolation frequency* *RD fk Isolation relative density*				

Highest isolation frequency *(Fr)* and relative density were observed for *Fusarium* genus, which made up 22.1%and 59.8% respectively. For the genus *Alternaria* isolation frequency *(Fr)* was 3.2% and isolation relative density *(RD)* 8.8%.

The lowest isolation frequency *(Fr)* and isolation relative density *(RD)* of fungi was detected for the genera *Cladosporium* and *Nectria* , which amounted to 0.3% and 0.9% respectively.

Thereby, molecular-genetic identification using analysis of the nucleotide sequences of the ITS region made it possible to perform identification of pathogens of potato and tomato diseases, which collected under production storage Also, fungi *Clonostachys* and *Acrostalagmus* genera were isolated, which are not pathogens of potato and tomato diseases.

CONCLUSION

In conclusion, 276 infected potato tubers and

tomato fruits were used to identify phytopathogenic fungi, which were collected from production storages. Molecular-genetic identification of microorganisms based on the analysis of the nucleotide sequence of the ITS region made it possible to taxonomically identify 102 isolates of phytopathogenicfungi, which belong to 7 genera. It was revealed, that the proportion of fungi, which belong to the genera*Fusarium* (60%), *Penicillium* (15%) and *Clonostachys* (12%), *Alternaria* (9%) were prevailed in structure.

The Isolation frequency*(Fr*) and relative density *(RD)* of 7 phytopathogenic fungi genera were calculated according to the results of molecular-genetic identification of isolates.Among the studied samples, isolation frequency of fungi genera ranged from 0.3% to 22.1%. Relative density of fungi species ranged varied 0.9% to 59.8%.

Thereby, molecular-genetic identification based on the analysis of the nucleotide sequences of the ITS region made it possible to perform identification of pathogens of potato and tomato diseases, which collected from storage. Also, fungi *Clonostachys* and *Acrostalagmus* genera were isolated, which are not pathogens of potato and tomato diseases.

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МОЛЕКУЛАЛЫҚ-ГЕНЕТИКАЛЫҚ САРАПТАМА НЕГІЗІНДЕ ӨНДІРІСТІК ҚОЙМАЛАРДАН ЖИНАЛҒАН КАРТОП ПЕН ҚЫЗАНАҚТА КЕЗДЕСЕТІН САҢЫРАУҚҰЛАҚ АУРУЛАРЫНЫҢ ҚОЗДЫРҒЫШТАРЫН ИДЕНТИФИКАЦИЯЛАУ

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ТҮЙІН

Мемлекеттер арасында картоп пен қызанақ көкөністерінің экспорты мен импортының кеңеюі, бүкіл әлемде фитопатогендік саңырауқұлақтардың зиянды түрлері мен штаммдарының тез таралуына ықпал етеді. Осыған байланысты фитопатогендік саңырауқұлақтарды анықтау ауыл шаруашылығы үшін маңызды. Қазіргі уақытта молекулалық-генетикалық әдістер өсімдіктер ауруының қоздырғыштарын диагностикалау мен анықтауда заманауи, перспективалы және микроағзалардың биологиялық ерекшеліктерін ескермей, идентификациялауға мүмкіндік беретіні дауыссыз. Ядролық рибосомалық гендер (ITS) аймағының нуклеотидтер тізбегін анықтау әдісі фитопатогендерді идентификациялауда, таксономиялық жіктеуде кеңінен қолданылады.

Фитопатогендік саңырауқұлақтардың изоляттарын бөліп алу үшін өндіріс қоймаларынан зақымданған картоп түйнектері мен қызанақ жемістерінің 276 данасы жиналды. Микроағзалардың молекулалық-генетикалық идентификациясы барысында ITS аймағының нуклеотидтер тізбегін анықтау негізінде бөлініп алынған 102 изолят 7 туысқа және 18 түрге жіктелді. Туыстық құрамы бойынша*Fusarium (60%), Penicillium (15%)*және *Clonostachys (13%), Alternaria (9%)*саңырауқұлақтарының үлесі басым екендігі анықталды.

Негізге алынған изоляттардың молекулалық-генетикалық идентификациялау нәтижелері бойынша фитопатогендік саңырауқұлақтардың 18 түрінің бөліп алу жиілігі *(Fr)* және салыстырмалы тығыздығы *(RD)* есептелді. Зерттелген үлгілердің ішіндегі саңырауқұлақтарды бөліп алу жиілігі 0,4%-тен 14,8%-ке дейін өзгерді. Оқшауланып алынған изоляттардың салыстырмалы тығыздығы 0,9%-ден 40,1%-ке дейін өзгерді.

Осылайша, ITS аймағының нуклеотидтер тізбегін анықтау негізінде молекулалық-генетикалық идентификациялау картоп пен қызанақ ауруларының қоздырғыштарын анықтауға мүмкіндік берді.

Негізгі сөздер: Картоп, қызанақ, саңырауқұлақ, ITS аймағы, идентификациялау, саңырауқұлақтарды бөліп алу жиілігі, саңырауқұлақтарды бөліп алудың салыстырмалы тығыздығы.

ИДЕНТИФИКАЦИЯ ВОЗБУДИТЕЛЕЙ ГРИБНЫХ БОЛЕЗНЕЙ КАРТОФЕЛЯ И ТОМАТА, СОБРАННЫХ В УСЛОВИЯХ ПРОИЗВОДСТВЕННЫХ ХРАНИЛИЩ, НА ОСНОВЕ МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКОГО АНАЛИЗА

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АБСТАКТ

Расширение масштабов экспорта и импорта урожая картофеля и томатов между странами способствует быстрому распространению наиболее вредоносных видов и штаммов фитопатогенных грибов по всему миру. В связи с этим, идентификация фитопатогенных грибов важна для сельского хозяйства. В настоящее время наиболее современными и перспективными способами диагностики и видовой идентификации возбудителей болезней растений являются молекулярно-генетические методы, позволяющие идентифицировать микроорганизмы, не имея представлений о его биологических особенностях. Одним из зарекомендованных методов для идентификации и таксономической классификации фитопатогенов является определение нуклеотидной последовательности участков ядерных рибосомных генов (ITS).

Для идентификации изолятов фитопатогенных грибов использовали 276 пораженных клубней картофеля и плодов томата, которые были собраны с производственных хранилищ. Молекулярно-генетическая идентификация микроорганизмов на основе анализа нуклеотидной последовательности ITS-региона позволила провести таксономическую идентификацию 102 изолятов фитопатогенных грибов, которые принадлежат к 7 родам. Было выявлено, что доля грибов рода *Fusarium (60%)*, *Penicillium (15%)* и *Clonostachys (12%)*, *Alternaria (9%).* Они доминировали в структуре родового состава.

На основе результатов молекулярно-генетической идентификации выделенных изолятов рассчитана частота изоляции *(Fr)* и относительная плотность *(RD)* 7 родов фитопатогенных грибов. Частота изоляции *(Fr)* варьировала от 0,3% до 22,1%. Показатели относительной плотности варьировали от 0,9% до 59,8%.

Таким образом, молекулярно-генетическая идентификация на основе анализа нуклеотидной последовательности ITS-региона позволила провести идентификацию возбудителей болезней картофеля и томата, собранных в условиях производственных хранилищ. Также были выделены грибы родов *Clonostachys* и *Acrostalagmus* , которые не являются возбудителями болезней картофеля и томатов.

Ключевые слова: картофель, томат, гриб, ITS регион, идентификация, частота изоляции грибов, относительная плотность изоляции грибов.

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