Original article

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FIRST REPORT OF *ALTERNARIA ALTERNATA* AND *RHIZOPUS ARRHIZUS* CAUSING DROOPING AND DEATH ON SUNFLOWER SPROUTS (*HELIANTHUS ANNUUS*) IN KAZAKHSTAN

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ABSTRACT

This study presents the first confirmed report of *Alternaria alternata* and *Rhizopus arrhizus* as causative agents of seedling wilt and death in sunflower (*Helianthus annuus*) in Kazakhstan. During phytopathological examination of seeds from four sunflower hybrids, characteristic fungal colonies were identified through morphological and molecular methods, including ITS sequencing and PCR amplification of Alt a1 and calmodulin genes. Symptoms of brown spots, desiccation, and gray mold were observed, with re-inoculation tests confirming pathogenicity. Phylogenetic analysis revealed a 91-98% similarity with known *Alternaria* and *Rhizopus* species. This research emphasizes the importance of monitoring imported seed material and implementing integrated disease management strategies. The findings contribute to understanding phytopathogen diversity in the region and inform recommendations for disease prevention in sunflower cultivation.

Key words: *Helianthus annuus, Alternaria alternata, Rhizopus arrhizus, seedling wilt, Kazakhstan, phytoanalysis, ITS sequencing, fungal pathogens*

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1 INTRODUCTION

Sunflower (*Helianthus annuus*) is one of the key oilseed crops in Kazakhstan. Its productivity and oil quality are of significant importance to the country's agricultural sector. However, various phytopathogens, including fungi of the genera *Alternaria* and *Rhizopus*, can substantially reduce both yield and product quality. In recent years, interest has grown in studying the impact of *Alternaria alternata* and *Rhizopus arrhizus* on sunflower.

Alternaria alternata as a Sunflower Pathogen. *Alternaria alternata* is a widespread phytopathogen affecting numerous agricultural crops, including sunflower. In Kazakhstan, alternariosis manifests as brown lesions on leaves, stems, and capitula, leading to reduced photosynthetic activity and ultimately decreased yield. The disease is favored by warm and humid weather conditions. According to Syngenta, the species *A. zinniae*, closely related to *A. alternata*, produces dangerous mycotoxins such as alternariol and altenuene, emphasizing the importance of controlling this pathogen [1].

Alternaria alternata and other species in the genus *Alternaria* are major causative agents of foliar spots and other sunflower diseases. These infections are widespread globally, including in Russia, South Africa, China, Greece, and Iran. The fungi infect sunflower at all developmental stages, causing leaf spotting, capitulum rot, yield reductions of up to 80%, and lower oil content in seeds [2].

The identification of *Alternaria spp.* involves both morphological and molecular methods. Pathogens are cultured on selective media for morphological examination and further analyzed using molecular markers such as histone H3 and ribosomal RNA genes [3]. Sequencing of the ITS region of rDNA has enabled the identification of genetically diverse populations of these fungi [4].

Thus, *Alternaria spp.* represent a significant threat to sunflower crops, and their effective management requires an integrated approach utilizing modern scientific methods. Investments in breeding, diagnostics, and disease control will enhance sunflower resistance and reduce economic losses.

Rhizopus arrhizus and Its Impact on Sunflower. *Rhizopus arrhizus* (syn. *R. oryzae*) causes head rot in sunflower in various parts of the world, including Arizona, USA, and Hebei and Xinjiang provinces in China. The disease is characterized by water-soaked, dark brown lesions on capitula, leading to premature drying and yield loss [5]. It typically appears at the onset of maturity, with dark-brown rotting spots on the under-

side of heads, often affecting large areas. The tissues become hard and dry, with underdeveloped seeds, causing yield losses of up to 30% or more [6].

At the same time, this fungus has demonstrated stress-alleviating properties, including thermal stress mitigation, through the secretion of secondary metabolites (e.g., indole-3-acetic acid and salicylic acid), which promote plant growth and improve stress resilience. Endophytes enhance mineral uptake (Ca, P, Mg) and increase phenolic and flavonoid content, acting as antioxidants that protect plants from oxidative stress [7].

The use of specific primers such as ITS1/ITS4 has enabled accurate identification of *Alternaria* spp., *Fusarium* spp., and *Rhizopus* spp. This method allows for rapid and precise pathogen determination. For example, *Rhizopus arrhizus* has been identified in both China and the United States using this approach [8].

Effective sunflower disease management is possible through a combination of chemical and biological control methods supported by precise molecular diagnostics. The use of endophytic fungi such as *Rhizopus arrhizus* may represent a promising direction in sustainable agriculture. Developing integrated plant protection strategies will help minimize crop losses and enhance sunflower productivity.

The aim of this study is to identify and confirm the role of *Alternaria alternata* and *Rhizopus arrhizus* as causative agents of sunflower (*Helianthus annuus*) seedling wilting and death in Kazakhstan and to propose recommendations for their diagnosis.

2 MATERIALS AND METHODS

2.1 Seed Material

The seed material complied with the requirements of GOST 12044-93 “Seeds of Agricultural Crops. Methods for Determining Disease Contamination.” The study was conducted on seeds of four sunflower (*Helianthus annuus* L.) varieties collected from the North Kazakhstan region.

2.2 Mycological Analysis

For mycological examination, seeds were first washed under running tap water for 1-2 hours and then surface-disinfected with 96% ethanol for 1-2 minutes. Following disinfection, the seeds were rinsed with sterile distilled water and dried between sterile filter paper sheets. Ten seeds from each variety were placed in Petri dishes containing Potato Dextrose Agar (PDA) medium and incubated at 25-27 °C for 7-10 days to promote fungal growth. The developing fungal colonies were examined in a drop of water under a Zeiss AxioScope A1 microscope for preliminary identification of phytopathogens.

2.3 Seed Germination Test

Germination was assessed using the filter paper roll method. Fifty seeds from each of the four samples were washed under running water for 20-30 minutes and then disinfected in a 1% potassium permanganate ($KMnO_4$) solution for 10-15 minutes. After disinfection, seeds were rinsed thoroughly with sterile or freshly boiled water.

The seeds were arranged with their pointed ends facing downward on a moistened strip of filter paper, placed 2 cm from the upper edge. A second strip of moist filter paper was laid over the seeds, and the layers were covered with a plastic plate (correx) and rolled into a cylinder. The rolls were placed into glass vessels, covered with glass lids to allow partial air exchange, and incubated in a thermostat at 22–23 °C. Seedlings were visually inspected on the 10th day of incubation, with a follow-up examination on day 14 if necessary. To confirm the identification of gray mold pathogens, sporulating fungal colonies were microscopically examined in a drop of water.

2.4 Statistical Analysis

Statistical processing of experimental data was performed using standard descriptive statistical methods in Microsoft Excel. Quantitative indicators included frequency of fungal infection across different sunflower hybrids and growth stages.

3 RESULTS

Sunflower (*Helianthus annuus L.*) is the leading oilseed crop in Kazakhstan. It accounts for up to 70% of the sown areas occupied by oilseeds and 85% of the gross harvest. Therefore, monitoring seed quality for diseases is critical. In March 2024, a phytoanalysis of seeds of 4 sunflower hybrids of foreign selection was carried out before spring sowing. Sun-

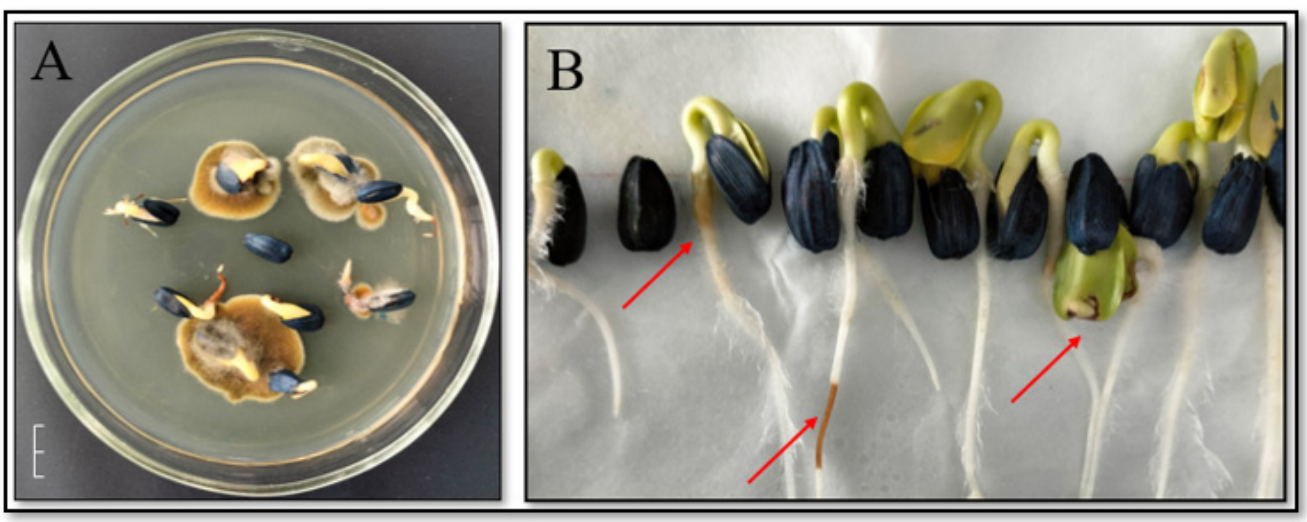

Figure 1 – Morphological characteristics and growth features of the causative agent of *Alternaria* spp.: A – colonies after 7 days at 28°C on potato dextrose agar; B – Growth results in rolls on the 7th day (arrows indicate lesion sites).

flower seeds of each hybrid (n = 15) were washed in 70% ethanol for 5 min, and rinsed three times with sterile distilled water. Seeds were placed on potato dextrose agar (PDA) and incubated at 28°C for 7 days. Gray-brown colonies grew from the seeds of one sunflower hybrid (Figure 1A). The sprouts of the seeds and leaves were brown and dried out (Figure 1A and 1B).

The fungus colonies had a light gray-whitish aerial mycelium. Conidia (n = 30) were pale to dark brown, and irregular and ellipsoid to ovoid conical. The conidia varied from 5 to 25 × 6 to 12 µm (n = 40) with longitudinal and transverse septations (Figure 2A and 2B).

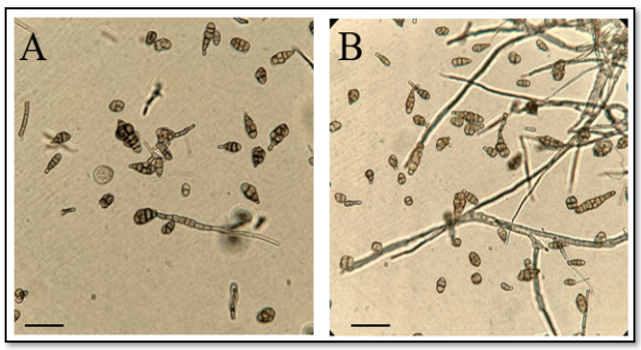

Figure 2 - Microscopic characters of *Alternaria* spp. (mycelium and conidia) (A) and (B).

These morphological characters were previously described and corresponded to *Alternaria* spp. [9, 10]. Within 3 to 7 days from the seeds of the other three hybrids, mycelial colonies with abundant aerial growth (Figure 3A) and black sporangia emerged (Figure 3B) and were identified as *Rhizopus arrhizus* (*R. oryzae*) on the basis of the presence of pale brown sporangiospores with bluish stripes [11]. The sporangia were black, subglobose, and 42 to 178 µm (n = 30) in diameter. The sporangiospores were unequal, globose or subglobose, and about 3.4 to 8.5 × 4.4 to 12.1 µm (n = 30) in diameter.

According to the results of the mycological analysis, out of a total of 120 isolates, 57.5% (n = 69) were identified as *Alternaria alternata*, 31.7% (n = 38) as *Rhizopus arrhizus*, and the remaining 10.8% (n = 13) belonged to saprophytic fungi (e.g., *Penicillium*, *Aspergillus*). The difference in the isolation frequency between *Alternaria* and *Rhizopus* was statistically significant.

Genomic DNA was extracted from mycelium using the liquid nitrogen and phenol-chloroform extraction method. A 568-bp product of the Alt a1 gene and a 472-bp product of the calmodulin protein-coding gene in *Alternaria* pathogens were amplified using the primer pairs Alt-for/Alt-rev [12]and CALDF1/CALDR1, respectively [13]. A 750 bp product of the ITS region on rDNA in *R. arrhizus* was amplified using following primer pairs ITS1/ITS4 [11]. The PCR reaction was done in a SimpliAmp thermal cycler under the recommended conditions. The sequencing was done using BigDye® Terminator v3.1 Cycle Sequencing Kit and the sequence was deposited in GenBank with accession nos. PP806899.1, PP943179.1, and PP502936.1. The results showed that the sequences of isolates were identical and had 91-98% identity with those of related species (Figure 4-5).

The alignment was made based on the sequence of Alt a1, different Alternaria species, 6 different isolates derived from *A. alternata* and *A. tenuissima* formed the largest clade with 98% similarity. *A. solani, A. cretica* and *A. grandis* formed a small clade with 91% similarity. The isolates studied in this work originated from a split clade with 98% similarity, suggesting high genetic similarity between the two species.

The alignment was based on the ITS1 sequence for the species *Rhizopus arrhizus*. The sample under study separated from the last ancestor in sequence with a similarity of 91%. The species *R. microsporus, R. delemar* and *R. americanus*, which were added as reference isolates, formed a small clade with a similarity of 74%.

The phylogenetic analysis was carried out with MEGA 11 [14]. To confirm the pathogenicity, week-old asymptomatic sprouts from healthy seeds were inoculated with a suspension of conidia by nebulization inoculation (1.0×106 conidia or spores/ml; obtained from 2-week-old cultures). Control plants were inoculated with sterile distilled water. The inoculated plants were placed in climate chamber and incubated for 10 days at 28°C. Symptoms were developed in leaves and roots of inoculated plants after 5 days. Symptoms of sprouts and leaves were similar to those observed on naturally infected plants, while control plants remained asymptomatic. Re-isolation of the pathogen confirmed the presence of the *A. alternata* and *R. arrhizus* pathogens. To our knowledge, this is the first report that sunflower seeds imported for cultivation in Kazakhstan are infected with alternariosis and head rot pathogens. The results can provide the basis for tightening the rules for importing sunflower seed into the country.

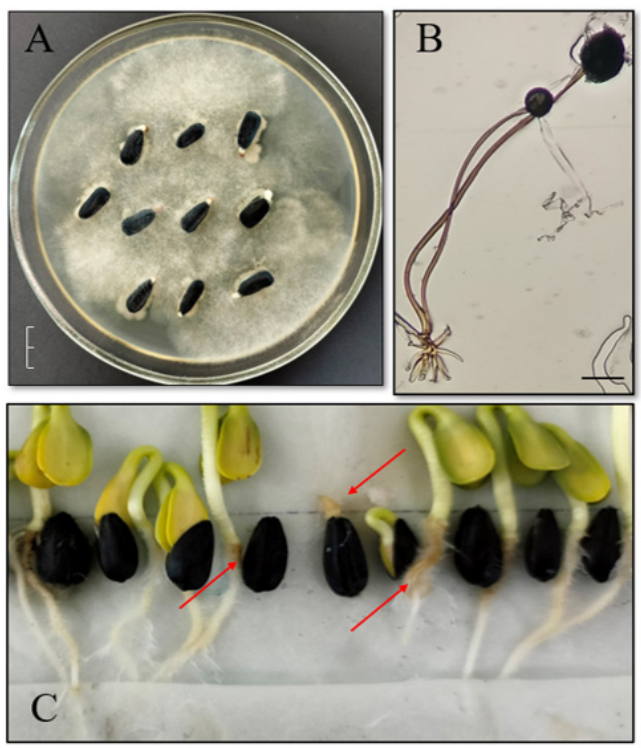

Figure 3 – Morphological characteristics and growth features of the causative agent of *Rhizopus arrhizus*: A – colonies after 7 days at 28°C on potato dextrose agar; B – microscopic characters of *R. arrhizus* (mycelium and sporangiospores); C – Growth results in rolls on the 7th day (arrows indicate lesion sites).

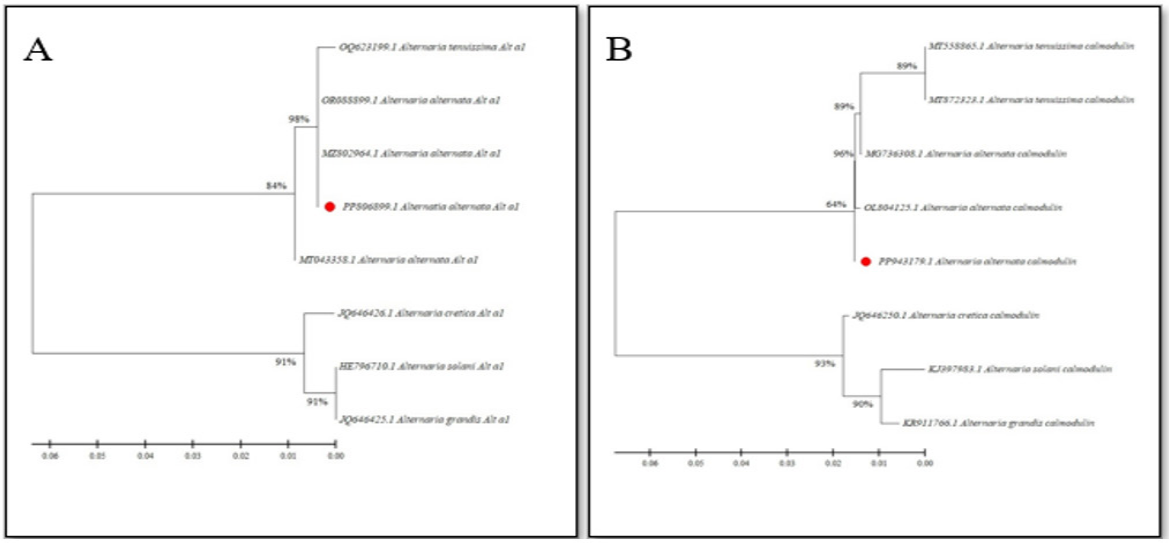

Figure 4 – Phylogenetic analysis (tree) generated with using marker genes: A – *Alternaria* spp. Alt a1 and B – calmodulin. Red circle – isolates from this study.

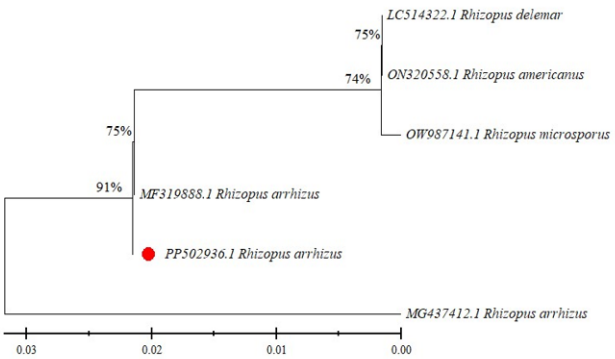

Figure 5 – *Rhizopus* spp. ITS. Red circle – isolates from this study.

4 DISCUSSION

The fungus *Alternaria alternata* is widely distributed in agroecosystems worldwide and is responsible for significant yield losses in sunflower. A study by Zhang et al. (2020) conducted in northern provinces of China revealed that *A. alternata* causes leaf spot and necrosis, resulting in yield reductions of up to 35%. The authors also confirmed the high aggressiveness of isolates and their resistance to certain fungicides [15]. Similar results were obtained in India, where pronounced morphological differences were observed among *Alternaria spp.* isolates from various regions, indicating genetic diversity and adaptation to environmental conditions [16].

Although *Rhizopus arrhizus* is a well-known phytopathogen, particularly under high humidity conditions, recent studies have demonstrated its potential for use in biotechnology. For instance, an isolate of *R. arrhizus* obtained from maize exhibited antagonistic activity against other plant pathogens (*Fusarium oxysporum*, *Sclerotinia sclerotiorum*) and stimulated plant growth through the production of enzymes and phytohormones. This highlights the potential of endophytic strains of this fungus as components of sustainable agriculture when properly selected and managed [17].

Modern approaches to fungal pathogen identification rely on whole-genome and ITS-based analyses. According to recent findings, amplification and sequencing of the ITS region of fungal DNA have demonstrated high accuracy in differentiating *Alternaria* species, particularly in mixed infections [18]. For *R. arrhizus*, the effectiveness of a multigene approach using ITS, LSU, and ACT markers has also been confirmed, which helps avoid misidentification [19]. These findings underscore the importance of comprehensive diagnostics in sunflower seed quality control systems.

5 CONCLUSION

This study documents the first detection of *Alternaria alternata* and *Rhizopus arrhizus* on sunflower sprouts in Kazakhstan, confirming their pathogenicity through molecular and morphological analyses. The results underscore the necessity of vigilant seed screening, especially for imported materials, and suggest adopting integrated diagnostic and control strategies to protect local sunflower production from these significant fungal threats.

AUTHOR CONTRIBUTIONS

RU, IO, ET, TS: conceptualization, study design, data curation, and writing – original draft preparation. IO, ET: collection of biological material. RU: data curation and laboratory experiments. RU, IO, ET: data analysis. TS: funding acquisition. RU, IO, ET, TS: editing and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The author declare no conflict of interest.

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ПЕРВОЕ СООБЩЕНИЕ О ВЫЯВЛЕНИИ *ALTERNARIA ALTERNATA* И *RHIZOPUS ARRHIZUS* КАК ВОЗБУДИТЕЛЕЙ УВЯДАНИЯ И ГИБЕЛИ ПОБЕГОВ ПОДСОЛНЕЧНИКА (*HELIANTHUS ANNUUS*) В КАЗАХСТАНЕ

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АННОТАЦИЯ

В настоящем исследовании впервые подтверждена роль грибов *Alternaria alternata* и *Rhizopus arrhizus* как возбудителей увядания и гибели побегов подсолнечника (*Helianthus annuus*) в Казахстане. При фитопатологическом

анализе семян четырёх гибридов подсолнечника были выделены характерные грибные колонии, идентифицированные с использованием морфологических признаков и молекулярных методов, включая секвенирование ITS-региона и ПЦР-амплификацию генов Alt a1 и calmodulin. Были зафиксированы симптомы в виде бурых пятен, высыхания и серой плесени; повторная инокуляция подтвердила патогенность. Филогенетический анализ показал 91-98% сходство с известными видами *Alternaria* и *Rhizopus*. Исследование подчёркивает необходимость контроля качества импортируемого посевного материала и внедрения интегрированных стратегий защиты растений. Полученные результаты способствуют более глубокому пониманию биоразнообразия фитопатогенов в регионе и могут быть использованы для профилактики заболеваний подсолнечника.

Ключевые слова: *Helianthus annuus, Alternaria alternata, Rhizopus arrhizus, увядание проростков, Казахстан, фитосанитарный анализ, секвенирование ITS, фитопатогенные грибы.*

ҚАЗАҚСТАНДА КҮНБАҒЫС (*HELIANTHUS ANNUUS*) ӨСКІНДЕРІНІҢ СОЛУЫ МЕН ЖОЙЫЛУЫНА *ALTERNARIA ALTERNATA* ЖӘНЕ *RHIZOPUS ARRHIZUS* САҢЫРАУҚҰЛАҚТАРЫНЫҢ СЕБЕПКЕР ЕКЕНІ ТУРАЛЫ АЛҒАШҚЫ ҒЫЛЫМИ МӘЛІМЕТ

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ТҮЙІН

Бұл зерттеу Қазақстанда күнбағыстың (*Helianthus annuus*) өскіндерінің солуы мен жойылуына себепкер болатын *Alternaria alternata* және *Rhizopus arrhizus* саңырауқұлақтарының алғаш рет ғылыми түрде анықталғанын көрсетеді. Күнбағыстың төрт гибридінің тұқымына фитопатологиялық талдау жүргізу барысында морфологиялық және молекулалық әдістер арқылы, оның ішінде ITS аймағының секвенирлеуі және *Alt a1*, *calmodulin* гендерінің ПТР-талдауы арқылы, тән саңырауқұлақ колониялары анықталды. Өсінділерде қоңыр дақтар, қурау және сұр көгеру белгілері байқалды; қайта жұқтыру сынақты патогенділікті растады. Филогенетикалық талдау *Alternaria* және *Rhizopus* туыстас түрлерімен 91–98% ұқсастықты көрсетті. Бұл зерттеу импорттық тұқым материалының фитосанитарлық бақылауын күшейтудің және өсімдіктерді қорғаудың кешенді шараларын енгізудің маңыздылығын айқындайды. Алынған нәтижелер өңірдегі фитопатогендік әртүрлілікті тереңірек түсінуге және күнбағыс дақылының ауруларының алдын алуға негіз болады.

Кілт сөздер: *Helianthus annuus, Alternaria alternata, Rhizopus arrhizus, өскіннің солуы, Қазақстан, фитосанитарлық талдау, ITS секвенирлеуі, фитопатоген саңырауқұлақтар.*