

Kostyukova V.S., Master of Science, **the main author**, <https://orcid.org/0000-0002-8922-964X>
RSE on REM «Institute of Plant Biology and Biotechnology» CS MSHE RK, Almaty, st. Timiryazev
45,050040, Kazakhstan, valera.kostyukova.15@gmail.com
Pozharskiy A.S., PhD student, <https://orcid.org/0000-0002-2581-2860>
RSE on REM «Institute of Plant Biology and Biotechnology» CS MSHE RK, Almaty, st. Timiryazev
45,050040, Kazakhstan, aspozarsky@gmail.com
Kapytina A.I., Master of Science, <https://orcid.org/0000-0001-5029-1107>
RSE on REM «Institute of Plant Biology and Biotechnology» CS MSHE RK, Almaty, st. Timiryazev
45,050040, Kazakhstan, anastasiya.kapytina@mail.ru
Gritsenko D.A., doctor PhD., <https://orcid.org/0000-0001-6377-3711>
RSE on REM «Institute of Plant Biology and Biotechnology» CS MSHE RK, Almaty, st. Timiryazev
45,050040, Kazakhstan, d.kopytina@gmail.com

DETECTION OF FUNGAL PATHOGENS OF THE GENUS *MONILINIA* IN CULTIVATED APPLE ORCHARDS

ANNOTATION

Fungal pathogens of the genus *Monilinia* pose a significant threat to fruit production, particularly in temperate regions such as southeastern Kazakhstan. This study investigates the morphological, molecular, and pathogenic characteristics of mixed infections caused by *Monilinia fructigena* and *Monilinia polystroma*, two closely related species responsible for fruit rot in apple orchards. Samples from symptomatic apple fruits were collected in the Almaty region, and pure fungal cultures were isolated and characterized. Morphological examination of the isolates revealed typical *Monilinia* colony characteristics, including dense mycelial growth and sporulation patterns. Experimental inoculation of *Pyrus communis* cv. ‘Conference’ confirmed high pathogenic potential and aggressive lesion development within 24 hours post-inoculation. Molecular identification using species-specific primers targeting the LAC2 gene confirmed mixed infections in 8 out of 9 samples. This is the first documented co-occurrence of *M. fructigena* and *M. polystroma* in Kazakhstan. The findings emphasize the necessity of integrating morphological assessment with molecular diagnostics for accurate pathogen identification. The development and use of specific molecular markers enable early detection, species differentiation, and improved phytosanitary strategies to manage moniliosis in fruit crops.

Key words: *Monilinia*, *M. fructigena*, *M. polystroma*, PCR, fruit rot, molecular diagnostics, moniliosis, pathogenicity.

Introduction. Fungal pathogens of the genus *Monilinia* pose a serious threat to fruit crops, particularly apple (*Malus domestica*). These pathogens are the causative agents of moniliosis, a severe disease that leads to significant yield losses, reduced commercial fruit quality, and deterioration of overall tree health [18]. The disease manifests as fruit rot, flower and shoot wilting, negatively impacting orchard productivity and the economic viability of fruit cultivation.

The most common *Monilinia* species affecting apples include *M. fructigena*, *M. laxa*, and *M. fructicola* [2]. *Monilinia fructigena* is the most widespread species in Europe and Asia, infecting apples and other stone fruits. This pathogen causes brown rot and fruit mummification, facilitating its persistence and dissemination within orchards [19].

A recently identified invasive species in Europe, *Monilinia polystroma*, was first described in Japan [2], [19]. Morphologically similar to *M. fructigena*, this pathogen exhibits higher aggressiveness, making it a potentially greater threat to apple orchards [7]. In recent years, its spread has been recorded in Central and Eastern European countries, including Hungary, Croatia, and Poland, which necessitates further monitoring and investigation of its biology and ecology [5, 16, 17].

Changes in climatic conditions, such as warming and increased precipitation during critical phenophases, may enhance the spread and pathogenicity of *Monilinia* species and contribute to the expansion of invasive species [1, 15]. In this regard, comprehensive studies are particularly relevant, including molecular diagnostics, epidemiological analysis, and phytosanitary mapping of disease outbreaks.

Early and accurate identification of *Monilinia* species plays a key role in the control of moniliosis, as the choice of protective and preventive measures depends on the specific pathogen species [22]. Traditional diagnostic methods based on morphological characteristics are often hindered by the high degree of similarity between *Monilinia* species and by the variability of symptoms depending on environmental conditions. In this context, molecular approaches, such as the amplification of species-specific DNA regions using PCR, are not only more accurate but also allow for the detection of pathogens at early stages of infection – before visible symptoms of the disease appear. Currently, molecular methods, including PCR diagnostics, enable precise species identification, which is particularly important for detecting invasive species [6]. Given the high pathogenicity of *Monilinia* fungi, timely detection, species composition analysis, and pathogen distribution studies are essential. Identification and monitoring of these pathogens are key steps in developing effective disease management strategies and preventing outbreaks.

Moreover, the use of molecular genetic data enables phylogenetic analysis, the identification of intraspecific variability, and the tracing of pathogen migration routes. This is particularly important for assessing phytosanitary risks associated with the introduction and spread of new genetic lineages of pathogens in agroecosystems [21].

This study aims to detect and molecularly identify the fungal pathogens *M. fructigena* and *M. polystroma* in cultivated apple orchards. The obtained results will refine the species composition of moniliosis pathogens in the studied regions. These findings may contribute to improving phytosanitary monitoring strategies and developing effective protective measures for fruit crops against moniliosis.

Materials and methods. *Collection of samples and cultivation of fungal phytopathogens.*

Samples of the cultivated apple variety “Aport” were collected from orchards in the Almaty region (43.364223, 77.478391). When typical symptoms of *Monilinia spp.* infection were detected, the affected fruits were collected for molecular genetic analysis. Fungal isolates were obtained and preserved following the method described in [10], with some modifications. Specifically, a sterilized wire loop was used to transfer conidia from the surface of the fruit (or mummified fruit) onto the surface of potato dextrose agar (PDA) in Petri dishes. The cultures were incubated at 25°C for seven days, with daily monitoring of fungal mycelium development. Subsequently, two successive transfers of homogeneous mycelium onto fresh nutrient media were performed to obtain pure cultures. The fungal pathogen collection was stored at 4°C.

Determination of the Aggressiveness of the Studied Isolates

To assess the virulence of the fungal isolates under study, a biological assay was conducted on fruits of the ‘Conference’ pear variety grown in the study region. The experiment was carried out following a modified version of the method adapted from protocol [12]. As experimental material, fruits without visible signs of physiological disorders or pathogen damage were selected. Preliminary sterilization included sequential treatment with soapy water, 70% ethanol, and double rinsing with sterile distilled water. After complete drying, a longitudinal incision 1–1.5 cm in length was made in the central part of each fruit using a sterile scalpel. Mycelial fragments obtained from four-day-old pathogen cultures were aseptically applied to the mechanically wounded areas. To avoid contamination by exogenous microorganisms, the inoculation sites were sealed with sterile tape. Fruits subjected to the same treatment without inoculum application served as the control. All samples were incubated in a sealed chamber at a temperature of 23–25 °C, relative humidity of 80–90%, and a 12-hour light/dark cycle. Upon completion of the seven-day incubation period, microscopic examination of the morphological features of fungal colonies that developed on the surface and within the fruit tissues was performed.

Molecular-Genetic Species Identification of Monilinia spp. DNA extraction from pure fungal cultures was performed using the commercial “Plant/Fungi DNA Isolation Kit” (Norgen) following the manufacturer’s protocol, with liquid nitrogen used for tissue homogenization. Identification of the moniliosis pathogen was carried out using conventional PCR [3]. The PCR mixture contained 1 U Taq DNA Polymerase (New England Biolabs), 1X Taq buffer, 0.2 μM dNTPs, 0.2 μM of each primer, and 20 ng of DNA template. The PCR conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, an optimized annealing temperature (Table 1) for 30 s, and 72°C for 40 s, with a final elongation step at 72°C for 4 min. PCR product analysis was performed using 2% agarose gel electrophoresis in TAE buffer.

Table 1 – Sequences and annealing temperatures used in the study

Determined species	Sequence 5’-3’	T _m , °C	Source
	F: TGCTACTCAAGTAAGTTGATCTGC	59	

<i>Monilinia fructicola</i>	R: TCCATCGCCGTATTGAAGT		Wang и др. 2018, [3]
<i>Monilinia fructigena</i>	F: TCAATCGATACCAACTGGTACGAT	62	
	R: TTCTCAACTGAAAGCCAATATTCTTTAG		
<i>Monilinia laxa</i>	F: AATTGATACCAACTGGTACGATGTG	59	
	R: AAAGCCAATATTCTTTGATATCAAGTTAGTG		
<i>Monilia polystroma</i>	F: ATACCAACTGGTACGATGTTACTCCTAC	56	
	R: AAGCCAATATTCTTTATATAAAGTTAGCGC		
<i>Monilia mumecola</i>	F: AAGTTGCATCCCTTCGCTGT	56	
	R: ATGACCAGGGGCATCTGTAATT		
<i>Monilia yunnanensis</i>	F: TCATTGGTAAGTTGCATCCCC	56	
	R: GATGACCAGGGGCATCTATAATTATT		

Results and Discussion. Fungal diseases of fruit crops, particularly those caused by representatives of the genus *Monilinia*, remain a significant concern in horticulture and fruit production in many countries, including Kazakhstan. Moniliosis (or fruit rot) substantially reduces yield, affects fruit quality, and causes premature fruit drop, resulting in economic losses for the agricultural sector. In recent years, an increase in the frequency of mixed infections has been observed, complicating diagnostics and necessitating a clearer understanding of pathogenesis, as well as the interactions between co-infecting species.

Of particular interest are cases of simultaneous presence of *M. fructigena* and *M. polystroma*, as both species are phylogenetically related, morphologically similar, yet differ in several traits, including virulence, sporulation structure, and sclerotia formation. The relevance of studying such infections lies not only in the increased aggressiveness of the pathogens but also in the need to develop differential diagnostic approaches. The present study focuses on the morphological and molecular characterization of such co-infections, as well as the assessment of their pathogenic potential.

In the present study, nine apple fruits exhibiting characteristic symptoms of moniliosis were analyzed. The samples ranged from those with isolated lesions to fully mummified fruits and were collected from apple orchards in the Almaty region. The infected tissue was used to obtain pure fungal cultures (Figure 1). During culture growth, white and grayish colonies with entire or slightly undulating margins were observed. Initially, a sparse aerial mycelium developed into a dense structure with concentric zonation. In some isolates, the colony margins darkened over time, and black stromatic plates appeared. The intense formation of these plates is a characteristic feature of *M. polystroma* [10].

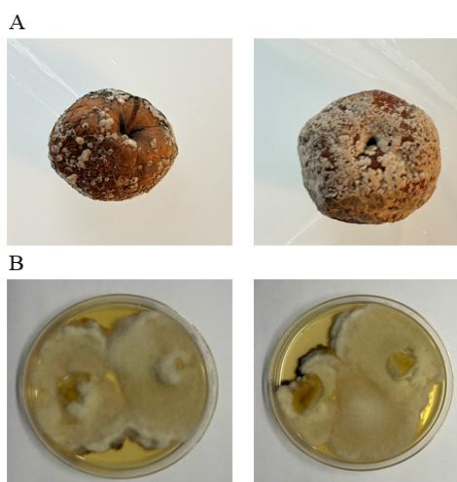


Figure 1A – Apples affected by moniliosis; Figure 1B – Cultures isolated from apple fruits with mixed infection.

Both species, *M. polystroma* and *M. fructigena*, exhibit the morphotype typical of the genus *Monilinia* – the formation of brown necrotic lesions on fruit, covered with pale beige to light brown sporulation cushions (conidial sporodochia). However, *M. polystroma* is characterized by more abundant and diffuse sporulation, often forming multiple concentric rings of sporodochia, whereas *M. fructigena*

displays sporulation in the form of individual, larger cushions arranged less regularly [4]. Due to the detection of a mixed infection, we were unable to observe distinct morphological differences between *Monilinia* colonies.

A significant distinguishing feature lies in the morphology of the sclerotia – compact resting structures. *M. polystroma* produces a substantially greater number of small sclerotia (up to 60–80 per fruit), which are distributed either diffusely or in a ring-like pattern, while *M. fructigena* forms a limited number of larger sclerotia (no more than 10–15 per fruit) [8]. No sclerotia were detected on fruits collected during the present study; therefore, this morphological trait has limited value for identifying mixed infections of moniliosis.

To assess the pathogenic activity and aggressiveness of isolates representing a mixed infection of *M. fructigena* and *M. polystroma*, an experimental inoculation was carried out on fruits of *Pyrus communis* cv. ‘Conference’ (Figure 2). Brown necrotic lesions appeared on the fruit surface within the first 24 hours after inoculation, preceding the sporulation phase. Lesion size was determined by measuring two orthogonal diameters, which ranged from 15 × 10 mm to 45 × 52 mm. This wide range is likely due to both intraspecific variability among isolates and the interaction between components of the mixed infection, as well as specific incubation conditions. Concentric zones of sporulation were visible on the affected tissues, typical of *M. fructigena*, represented by conidial pustules of varying color – from white to light and dark brown – depending on the maturity of the sporulating structures.



Figure 2 – The condition of pear fruits on the 7th day of incubation after infection with isolated isolates: A – sample, B – negative control

Microscopically, and consistent with the literature, both species form oval or ellipsoidal conidia that are uninucleate, hyaline, and smooth-walled. On average, conidia of *M. polystroma* are slightly smaller in size [13]. However, there is partial overlap in size ranges, making precise identification based solely on microscopic traits difficult. In the conidial stage of development, we observed ellipsoidal conidia forming in branched chains (Figure 3). Morphological analysis revealed a predominance of oval and lemon-shaped conidia. By the fourth day of cultivation, intensive mycelial growth was observed, accompanied by active conidiogenesis and pronounced sporulation, indicating a high epiphytotic potential of the pathogen under natural infection conditions.

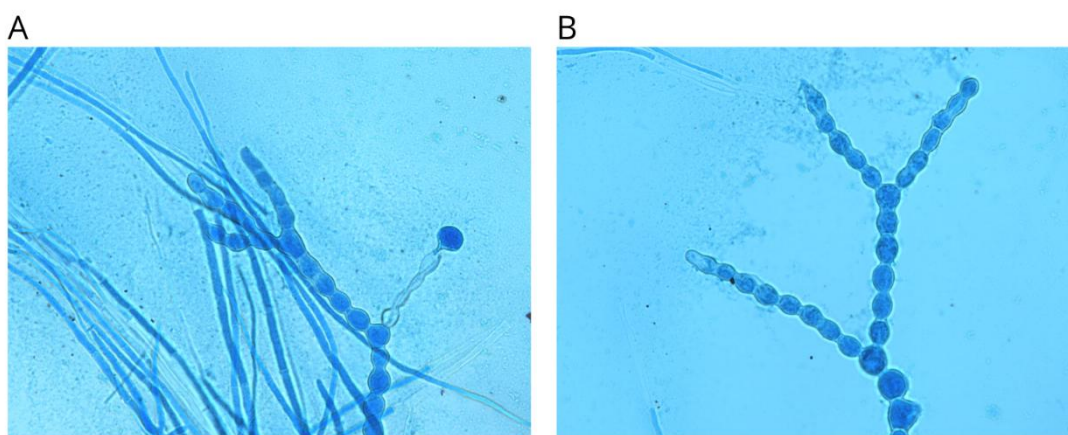


Figure 3 – Conidial structures on the surface of inoculated pears on days 4 (A) and 7 (B) of incubation

The simultaneous presence of *M. fructigena* and *M. polystroma* on the same plant has not been previously reported worldwide. Both phytopathogenic species share similar environmental conditions for development and infection mechanisms, producing conidiophores and conidia on the surface of infected fruits, which facilitate a high degree of infection [20], [11]. However, the morphological similarity between these species complicates diagnosis when infecting on the same substrate, necessitating the use of molecular methods. Polymerase chain reaction (PCR) is a highly sensitive technique that enables pathogen detection based on species-specific DNA sequences. Cultures obtained from nutrient media were subsequently used for molecular-genetic species identification.

For molecular-genetic diagnostics, species-specific primers complementary to the variable region of the constitutive laccase-2 (LAC2) gene, a key virulence factor, were used [3]. The analysis confirmed a mixed infection with *M. fructigena* and *M. polystroma* in 8 out of 9 samples, while *M. fructigena* alone was detected in one sample (Figure 4).

M. polystroma is morphologically and phylogenetically similar to *M. fructigena* and is often misidentified as a variant of the latter. However, molecular studies, including analyses of ITS regions of rDNA and the β -tubulin gene, have confirmed its distinct species status [9].

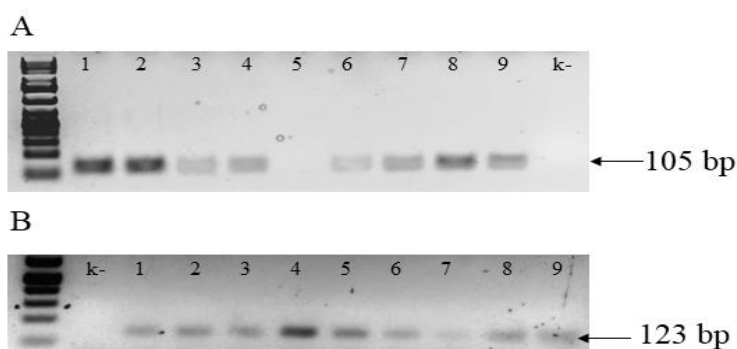


Figure 4 – Species identification results of *Monilinia* spp. A – Identification of *M. polystroma*, amplicon size: 105 bp; B – Identification of *M. fructigena*, amplicon size: 123 bp. The molecular size standard used: GeneRuler 1 kb Plus DNA Ladder (ThermoFisher Scientific).

During this study, a collection of fungal cultures was established, consisting of *M. fructigena* and *M. polystroma* isolates obtained from apple orchards in the Almaty region.

The co-occurrence of *M. fructigena* and *M. polystroma* represents a complex phytopathological phenomenon that necessitates precise molecular diagnostics [14]. PCR-based species-specific markers serve as a primary tool for detecting and monitoring these pathogens in agroecosystems. The development and implementation of more sensitive molecular techniques will enable more effective control of pathogen spread and the formulation of biological and chemical management strategies.

Thus, the results of the present study indicate a high prevalence and aggressiveness of mixed infections of *M. fructigena* and *M. polystroma* in apple orchards of southeastern Kazakhstan. The obtained data highlight the importance of combining morphological and molecular methods for the accurate diagnosis and monitoring of *Monilinia* pathogens. The development and implementation of specific diagnostic markers offer promising prospects for earlier detection of infection, species differentiation, and the application of targeted phytosanitary measures. Further research should focus on assessing the resistance of various fruit tree cultivars to these pathogens and exploring the potential for biological control under field agroecosystem conditions.

Conclusion. This study successfully detected and molecularly identified fungal pathogens of the genus *Monilinia* affecting apple (*Malus domestica*), with a particular focus on *M. fructigena* and *M. polystroma*. The results confirm the co-occurrence of these phytopathogens in apple orchards in the Almaty region, which has not been previously documented. The identified mixed infection highlights the complex nature of moniliosis pathogen spread and underscores the necessity for continued monitoring.

The use of molecular methods, particularly PCR diagnostics, has demonstrated high accuracy in species identification, reaffirming the importance of molecular approaches for phytosanitary monitoring and disease outbreak prevention.

This research contributes to the understanding of *M. polystroma* distribution in Central Asia and provides a foundation for developing improved strategies for the control and protection of fruit crops. Future studies should expand the analysis to additional regions and incorporate further molecular markers to enhance our understanding of the biology and ecology of these phytopathogens.

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ТҮЙІН

Monilinia туысына жататын саңырауқұлақ патогендері жеміс шаруашылығына, әсіресе Қазақстанның оңтүстік-шығысындағы қоңыржай аймақтарда елеулі қатер төндіреді. Бұл зерттеу алма бақтарындағы жеміс шірігін тудыратын, бір-біріне жақын екі түр – *Monilinia fructigena* мен *Monilinia polystroma* – себеп болатын аралас инфекциялардың морфологиялық, молекулалық және патогендік ерекшеліктерін қарастырады. Алматы облысынан ауру белгілері бар алма жемістері жиналып, таза саңырауқұлақ дақылдары бөлініп алынды және жан-жақты сипатталды. Морфологиялық зерттеу нәтижесінде изоляттардың *Monilinia* туысына тән колониялық белгілері анықталды, соның ішінде тығыз мицелий өсуі мен спора түзу ерекшеліктері байқалды. *Pyrus communis* ‘Conference’ сортына жүргізілген жасанды жұқтыру тәжірибелері 24 сағат ішінде жоғары патогендік мүмкіндігі бар екенін және залалдану ошағының жылдам дамитынын көрсетті. LAC2 геніне бағытталған түрге тән праймерлер қолданылған молекулалық талдау нәтижесінде 9 үлгінің 8-інде аталған екі түрдің аралас инфекциясы анықталды. Бұл – Қазақстанда алғаш рет *M. fructigena* мен *M. polystroma* түрлерінің қатар кездесуі тіркелді. Алынған нәтижелер патогендерді дәл анықтау үшін морфологиялық әдістерді молекулалық диагностикамен біріктірудің маңыздылығын айқын көрсетеді. Түрлерді ерте және нақты ажыратуға мүмкіндік беретін арнайы молекулалық маркерлерді әзірлеу мен қолдану монилияозға қарсы фитосанитарлық шараларды жетілдіруге ықпал етеді.

РЕЗЮМЕ

Грибные патогены рода *Monilinia* представляют значительную угрозу для производства фруктов, особенно в умеренных регионах, таких как юго-восток Казахстана. В данном исследовании изучаются морфологические, молекулярные и патогенные характеристики смешанных инфекций, вызванных *Monilinia fructigena* и *Monilinia polystroma* - двумя близкородственными видами, ответственными за гниль плодов в

яблоневых садах. Образцы с симптоматичными яблоками были собраны в Алматинской области, из них выделены и охарактеризованы чистые культуры грибов. Морфологическое исследование изолятов показало типичные для *Monilinia* характеристики колоний, включая плотный мицелий и особенности спороношения. Экспериментальное заражение сорта *Rugus communis* cv. 'Conference' подтвердило высокий патогенный потенциал и быстрое развитие поражений — в течение 24 часов после инокуляции. Молекулярная идентификация с использованием видоспецифичных праймеров к гену LAC2 подтвердила смешанные инфекции в 8 из 9 образцов. Это первое задокументированное совместное присутствие *M. fructigena* и *M. polystroma* в Казахстане. Полученные данные подчёркивают необходимость интеграции морфологической оценки с молекулярной диагностикой для точного определения патогенов. Разработка и использование специфичных молекулярных маркеров позволяют осуществлять раннее выявление, дифференцировать виды и улучшать фитосанитарные стратегии борьбы с монилиозом плодовых культур.