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SPECIES COMPOSITION OF FUNGI OF THE GENUS *Fusarium* Link ON GARLIC PLANTS IN MOSCOW REGION

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Abstract

Garlic (*Allium sativum* L.) cultivation is restrained by the lack of seed propagation and high sensitivity to pathogenic fungi, bacteria, viruses and nematodes. Currently, *Fusarium* dry rot caused by soil fungi of the genus *Fusarium* is the most harmful disease of the crop. For the first time, in a multidisciplinary study, we have quantified the ratio of the main *Fusarium* dry rot pathogens affecting garlic in the Moscow region. Data shows the percentage of occurrence of key pathogens, high harmfulness and a dominant position of dry rot of the species *F. proliferatum* in the complex of pathogens. The work aimed to investigate the species composition of plant pathogenic fungi of the genus *Fusarium* on a garlic (*Allium sativum* L.) crop in the Moscow region. Diseased bulbs ($n = 1108$) of winter garlic cv. Gladiator were collected during 2019 to 2021 (All-Russian Research Institute of Vegetable Growing) and divided into sample sets based on the same symptoms of bulbs and cloves damage. Two hundred garlic cloves from each sample were surface sterilized by common methods and used to isolate pathogens. Scrapings from damaged clove tissues were placed on Czapek's medium in sterile Petri dishes and incubated for 12 days at 25 °C. The colonies were subcultured until monospore cultures were obtained. The isolates were identified using taxonomic keys. The frequency of occurrence of pathogens was calculated. Analysis of the marker sequences of the *TEF1α* gene (translation elongation factor 1 alpha) (~ 550 bp) and *MCM7* (gene encoding minichromosome maintenance protein 7) (~ 650 bp) was performed to confirm the taxonomic identification of the isolates. Molecular identification of the isolates was performed by quantitative PCR using species-specific primers for *F. proliferatum*. NCBI's BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to analyse the nucleotide sequences of the *TEF1α* and *MCM7* genes. Seven genera of fungi, the *Fusarium*, *Penicillium*, *Botrytis*, *Embellisia*, *Aspergillus*, *Alternaria*, and *Sclerotium* were isolated from the garlic bulbs with varying degrees of occurrence. Among these pathogens, the genus *Fusarium* prevailed with a frequency of more than 44 %. Of six identified species, the *F. proliferatum*, *F. oxysporum*, *F. poae*, *F. verticilloides*, *F. culmorum* and *F. acuminatum*, the *F. proliferatum* predominated at a frequency of 61.4-75.6 %. Sequencing of DNA markers confirmed identification of the *F. proliferatum* isolates. The similarity to the sequences deposited in the NCBI database was 99-100 % for *TEF1α* gene (reference sequence numbers MN158137, KP267240, MN012923, KT224299) and 98-99 % for *MCM7* gene (reference sequence numbers XM031230017, XM0311M36726). qPCR analysis using specific primers also confirmed these results. Our findings add on the available data on the prevalence and dynamics of changes in the species composition of fungi of the genus *Fusarium* in the Moscow region. The data are also practically important for the development of more effective methods to prevent and control

Fusarium diseases of garlic.

Keywords: garlic, *Fusarium genus*, *Fusarium* dry rot, frequency of occurrence, DNA markers, polymerase chain reaction

Garlic (*Allium sativum* L.) is a vegetatively propagated plant which leads to the gradual accumulation of pathogens and their transmission to offspring. Despite the fact that garlic has bactericidal properties, it itself is susceptible to fungal, bacterial, nematode and viral diseases. The greatest harm is caused by fungi that accumulate in the tissues of planting material, which leads to a decrease in yield and degeneration of varieties [1-3].

Garlic diseases caused by representatives of the genus *Fusarium* Link which are called dry bottom rot are among the most harmful and common. In some years, the frequency of occurrence of *Fusarium* wilt on garlic reaches 10-80%, which causes yield losses from 17 to 60% [3-6]. Currently, the genus Fusarium includes more than 300 species [7-9]. Among the species characterized by the ability to infect garlic, *F. oxysporum* and *F. solani* are the most common [10, 11].

To date, the State Register of Breeding Achievements of the Russian Federation [12] does not contain garlic varieties that are resistant to fungi of the genus *Fusarium*. This is largely due to the high plasticity of the latter and the ability to remain viable for several years, form chlamydospores or sclerotia, and acquire resistance to chemical or biological fungicides [13-15].

The composition of pathogens varies depending on the host plant, agrotechnical and meteorological growing conditions. The weather conditions of the Moscow region are favorable both for the growth of garlic plants and for the development of fungi of the genus *Fusarium*, and therefore in some years there is a high infection of plants with *Fusarium* pathogens [16].

To identify fungi of the genus *Fusarium*, morphological and biological approaches together with DNA analysis and gene systematics are used. It is the combination of these methods that gives the most reliable results [7].

In this work, for the first time, a quantitative assessment of the ratio of the main pathogens of *Fusarium* dry rot affecting garlic in the Moscow region was carried out using a multidisciplinary approach. The percentage ratios of the occurrence of key pathogens are shown, high harmfulness and a dominant position in the complex of pathogens of dry rot of the species *F. proliferatum* are revealed.

The aim of the work was to study the species composition of phytopathogenic fungi of the genus Fusarium on a garlic culture in the Moscow region.

Materials and methods. The studies were carried out at the All-Russian Research Institute of Vegetable Growing, a branch of the Federal Scientific Center for Vegetable Growing (Ramensky District, Moscow Province), on winter garlic cv. Gladiator variety, included in the State Register of Breeding Achievements of the Russian Federation, approved for use since 2011 [12]. Plants were grown according to conventional methods [17]. In the period from 2019 to 2021, 1108 diseased bulbs were selected and divided into samples with the same symptoms of damage to the bulbs and cloves. To isolate pathogens, 200 cloves were taken from each sample and surface-sterilized [18].

After sterilization, scrapings were made from infected tissues, the material was plated on Czapek's medium in Petri dishes with and incubated at 25 °C for 12 days.

Pathogen colonies were serially subcultured until a monospore culture was obtained. The isolates were then classified using the taxonomic keys of the *Fusarium* laboratory manual [19]. The frequency of occurrence of pathogens was determined by the formula: $A = B/C \times 100\%$, where B is the number of samples in which *F. proliferatum* was found, C is the total number of samples analyzed.

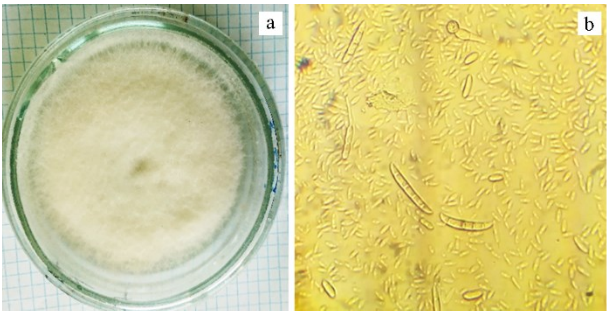
To confirm the taxonomic identification of the isolates, molecular genetic analysis of the marker sequences of the *TEF1*α gene (TEF30F: 5′-CGTCGTCATCGGCCACGT-3′, TEF650R: 5′-ACCAATGACRGTGACATAGTAGC-3′) and the *MCM7* gene (MCM7F: 5′-GCACCTGTCAGCTATGAGAAGC-3′, MCM7R: 5′-CAAGTTCCCTGCGTCCAC-3′) was performed, the primers were designed in this study.

DNA isolation and cloning of PCR products were performed according to the previously described procedure [20, 21]. For PCR amplification, a universal protocol was used: 90 s at 93 °C; 20 s at 93 °C, 5 s at 60 °C, 5 s at 67 °C (5 cycles); 1 s at 93 °C, 5 s at 64 °C, 5 s at 67 °C (40 cycles). DNA molecules were sequenced on an ABI PRISM 3730 automatic sequencer (Applied Biosystems, USA). To analyze the decoded nucleotide sequences of the *TEF1*α and *MCM7* genes, we used the BLAST algorithm on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi/).

DNA analysis of the isolates was also performed using quantitative PCR with *F. proliferatum* specific primers: FprolF — 5′-GTCCTCCCTCGAGACTGCC-3′, FprolR — 5′-GTTCTTCTTCGTGGAGTAGCCG-3′; fluorescent labeled probe 5′-(BHQ1)-ACGCAGACGT(FAMdT)CTTACAATCCCCCGAAA-3′. Amplification was carried out in a detecting cycler DT-96 (DNK-technology, Russia) in accordance with the following program: 90 s at 93 °C; 1 s at 93 °C, 5 s at 64 °C, 5 s at 67 °C (45 cycles).

Results. Our studies have shown that even if the necessary agrotechnical requirements are met, in some years up to 14.5% of garlic plants can be affected by fungal infections. The analysis of affected samples detected representatives of 7 genera of fungi (*Fusarium*, *Penicillium*, *Botrytis*, *Embellisia*, *Aspergillus*, *Alternaria*, *Sclerotium*) which occur with different frequencies. Among these pathogens, fungi of the genus *Fusarium* were the most common. During the growing season and storage, the frequency of their occurrence was more than 44%.

From garlic samples affected by *Fusarium*, 280 isolates belonging to 6 species were isolated: *F. proliferatum*, *F. oxysporum*, *F. poae*, *F. veticilloides*, *F. culmorum*, and *F. acuminatum*. The highest frequency of occurrence (from 61.4 to 75.6%) was characteristic of the species *F. proliferatu*m. The frequency of occurrence of *F. oxysporum* was lower and amounted to 15.1-32.3%. Fungi of the species *F. verticilloides* (9.3%), *F. poae* (4.4-7.7%), *F. culmorum* (1.9-5.1%) and F. *acuminatum* (2.6%) were less common.



A colony (a) **and spores** (b, Olympus CX33, Olympus Corporation, Japan) **of *Fusarium proliferatum* isolated from infected plants of winter garlic (*Allium sativum* L.) cv. Gladiator in the Moscow Province** (Chapek's medium, 12 days, 25 °C).

Microbiological analysis showed that the colonies of the fungus *F. proliferatum* on day 12 of growth on Czapek's medium had white mycelium, oval microconidia, 2.2-3.5×8.0-10.0 μm in size, slightly curved macroconidia with 3-5 septa, 3.3-4.1×30-46 μm in size (Fig.).

In molecular genetic analysis, genes of translation elongation factor 1 alpha (*TEF1*α) and minichromosome maintenance protein 7 (*MCM7*) were chosen as informative DNA markers to confirm the species of the studied strains. The

*TEF1*α gene is considered as the "gold standard" of molecular taxonomy of fungi of the genus *Fusarium*. The *MCM7* gene for the taxonomic classification of the genus *Fusarium* has not been practically used, but its taxonomic potential has been demonstrated using other fungi as an example [20].

Molecular genetic analysis of the marker sequences of the *TEF1*α and *MCM7* genes confirmed the identification of the studied strains as *F. proliferatum*. The correspondence with the sequences deposited in the NCBI database (https://www.ncbi.nlm.nih.gov/) was 99-100% for the *TEF1*α gene (reference sequence numbers MN158137, KP267240, MN012923, KT224299) and 98-99% for *MCM7* (XM03123 0017, XM031176796, XM31230017). Strain identification was also confirmed by qPCR with specific primers for *F. proliferatum* (Table).

Quantitative PCR analysis of *Fusarium proliferatum* isolated from infected winter garlic (*Allium sativum* L.) cv. Gladiator plants in the Moscow Province with a *F. proliferatum* specific test system

Изолят	Cq, Fam	Test result
Fo6	23.7	+
Fo5	25.4	+
Fo13	19.6	+
Fo10	16.5	+
Fo11	18.5	+
Fo2	17.8	+
Fo4	24.2	+
F09	17.8	+
Fo14	20.6	+
Fo1-1	19.8	+
Positive control	18.1	+
Negative control		–

Note. Cq — threshold cycle, "+" — positive control, "–" — negative control (water).

In previous studies, it was found that complexes of *F. oxysporum* and *F. solani* species are the most harmful and widespread on garlic in the Moscow region [3, 4]. Obviously, the dynamics of the species composition of fungi of the genus *Fusarium* can change depending on the natural agroecosystem and weather conditions during the year. However, the frequency of occurrence of the species *F. poae*, *F. culmorum*, *F. verticilloides*, and *F. acuminatum* on garlic culture in the Moscow region remains relatively stable.

Until 2021, there was no information about the damage of garlic by fungi of the species *F. proliferatum* in the Moscow region. However, it is known that abroad this pathogen is one of the most virulent, belongs to polyphages, capable of strongly infecting plants of different families [8], including garlic plants [22-25]. Garlic disease caused by *F. proliferaum* was first reported in 2002 in Germany [25], then in North America [23], Serbia [26], Italy [27], Spain [10, 28], India [29], Egypt [30], and France [31].

Thus, during the growing season in the conditions of the Moscow region, garlic plants can be affected by pathogenic fungi belonging to seven genera, *Fusarium*, *Penicillium*, *Botrytis*, *Embellisia*, *Aspergillus*, *Alternaria* and *Sclerotium* which occur with different frequencies. Among these pathogens, representatives of the genus *Fusarium* are the most common. The frequency of their occurrence was more than 44%. Within this genus, isolates belonging to the species *F. proliferatum*, *F. oxysporum*, *F. poae*, *F. verticilloides*, *F. culmorum*, and *F. acuminatum* were found. The highest frequency of occurrence (from 61.4 to 75.6%) was characteristic of the species *F. proliferatum*. Molecular genetic analysis of the marker sequences of the *TEF1*α and *MCM7* genes confirmed that the isolates belong to the species *F. proliferatum*. The similarity with the sequences deposited in the NCBI database was 99-100% for the *TEF1*α gene and 98-99% for the *MCM7* gene.

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