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Fungal diversity in tomato (*Solanum lycopersicum*) leaves and fruits in Russia

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ABSTRACT

Sequencing of cloned PCR-amplified species-specific rDNA fragments and isolation of axenic cultures from tomato fruits was carried out to study the mycobiota of tomato leaves and fruits in European part of Russia. DNA was extracted from the leaves, and library of ITS region fragments was constructed in *E. coli* by cloning of PCR products. This survey revealed fourteen species associated with disease-affected leaves: *Septoria lycopersici*, *Fulvia fulva (=Cladosporium fulvum)*, *Didymella glomerata (=Phoma glomerata)*, *Cladosporium herbarum*, *Podosphaera fusca*, *Neocamarosporium goegapense (=Phoma betae)*, *Rhizoctonia solani*, *Candida albicans*, *Dioszegia hungarica*, *Cladosporium cladosporioides*, *Didymella lycopersici*, *Alternaria infectoria*, *Alternaria alternata*, *Cryptococcus tephrensis*. In the leaves from healthy plants without any visible symptoms DNA of three species was found: *Aspergillus versicolor*, *Alternaria alternata*, *Aureobasidium pullulans*. Analysis of axenic cultures isolated from green diseased tomato fruits revealed fungal species: *Alternaria alternata*, *Alternaria solani*, *Phomopsis phaseoli*, *Fusarium equiseti*, *Chaetomium cochliodes*, *Clonostachys* sp., *Irpex lacteus*, *Colletotrichum coccodes*. This research provides new information on the mycobiota of tomato in Southern Russia, the main tomato producing region of the country.

Keywords: mycobiota of tomato leaves, phylloplane, plant pathogenic fungi, fungal species identification, tomato diseases, fungal diversity

INTRODUCTION

Climatic changes and the intensive exchange of seed material lead to the emergence of new types of fungi associated with tomato plants. Foliar diseases cause significant crop losses annually worldwide. Leaves can also provide niches for the excitants of fruit infections. For example, *Alternaria solani*, *Alternaria alternata*, *Phytophthora infestans*, *Colletotrichum coccodes* can cause leaf spots, stem canker and fruit rot. Among fungi associated with tomato plants, there are not only phytopathogens, but also saprotrophs and mycotrophs, which can protect the tomato from diseases and phytophages. On the other hand, pathogenic or allergenic to human's microorganisms can be in phylloplane too. Despite this, little is known about tomato leaves' mycobiota by now. Data from a metagenomic analysis shows that mycobiota of tomato phylloplane can be highly diverse. Fungal phyla identified from the surfaces of tomato leaves from Virginia, USA using 18S rRNA gene sequencing included the following large taxonomic groups: Ascomycota, Basidiomycota, Chytridimycota, Glomeromycota, Zygomycota. Dominant fungal genera identified on aerial surfaces were *Hypocrea*, *Aureobasidium*, and *Cryptococcus* (Ottesen et al., 2013). A high variety of fungi associated with tomato leaves was also noted in the work of Japanese researchers (Toju et al., 2019). Another method used for mycobiota analysis involves the isolation and analysis of pure fungal cultures. *Alternaria alternata*, *A. solani*, *Phytophthora infestans*,

Septoria lycopercisi were isolated from tomato leaves in Pakistan (Chohan et al., 2016). In the work of Monaco et al. (2001), it was shown that leaf position in the canopy influenced the fungal population structure. Some species, such as *Epicoccum nigrum*, *Chaetomium globosum*, *Aspergillus* sp., *Trichoderma harzianum*, *T. polysporum* and *Penicillium* spp., were more abundant on lower leaves, while *A. alternata*, *Cryptococcus luteolus*, *Rhodotorula* sp., *Pleospora herbarum*, *Fusarium semitectum*, *F. oxysporum* and *Cladosporium cladosporioides* were present in greater numbers on leaves located in the upper and medium levels (Monaco et al., 2001). However, both above methods have their drawbacks. NGS sequencing rarely allows identification of species of microorganisms. Isolation of axenic cultures does not make it possible to completely study the species composition, since not all fungi can grow on nutrient media. In this work the generation of clone libraries of full-length internal transcribed spacer region ITS1-5,8S-ITS2 was chosen as a powerful, culture-independent tool for observing fungal communities. This approach allows to identify fungal DNA up to the species level. The combined use of this method with the isolation and analysis of axenic fungal cultures made it possible to maximally reveal the mycobiota associated with tomato leaves and fruits.

MATERIALS AND METHODS

Analysis of leaves

Leaves with spots and necrosis were collected in July-August, 2014 by the authors themselves from field-grown plants in five regions of European Russia (Figure 1, Table 1). In total, samples from 4 commercial fields and 3 small private gardens were investigated. In Rostov region and Krasnodar territory samples were collected in commercial fields; in Voronezh region and Stavropol territory – from small kitchen-gardens. Leaves with no visible symptoms were collected from healthy tomato plants in small private garden in the national park "Kurshskaya kosa" in Kaliningrad region. Leaves were collected from the top third of each selected plant. Fresh leaves were fixed in 70% alcohol immediately after sampling. For analysis one simple leaf from one field or garden was taken.

In the laboratory leaf surfaces were washed with sterile water and dried. One simple leaf with small necrotic spots from every tested field was taken for analysis. DNA was extracted from the entire leaf using the conventional hexadecyl-trimethyl-ammonium bromide (CTAB) protocol with chloroform deproteinisation (Kokaeva et al., 2018a). DNA purity and concentration were measured using a NanoDrop™ spectrophotometer. The fungal-specific primer pair ITS1F and ITS4 (Gardes and Bruns, 1993; White et al., 1990) was used for the initial PCR from leaf material. After amplification, the whole PCR product was analyzed in a 1.5% agarose gel stained with ethidium bromide. Amplification products were removed from the agarose gel with a sterile scalpel and cleaned with Cleanup Standard kit (Evrogen, Russian Federation). Cloning of PCR products was carried out using a pAL2-T Vector System kit (Evrogen, Russian Federation) and dh5α competent cells. Sterile water without addition of fungal material served as a negative control.

Ligations were performed by mixing the PCR product with rapid ligation buffer, pAL2-T Vector, T4 DNA, and ligase. Bacterial transformations were performed by adding the ligation product to dh5α competent cells and spreading on LB-agar plates, each with addition of ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma), and IPTG (isopropyl β-D-1-thiogalactopyranoside) (Promega). Plates were incubated at 37 °C for 24 h. Bacterial cells carrying the vector with successfully ligated PCR product form white colonies under the conditions described above.

DNA was extracted from white colonies and ITS regions were amplified using M13 primers (M13f-TGTAAAACGACGGCCAGT/ M13r - CAGGAAACAGCTATGAC). PCR products were analyzed in 1.5% agarose gel to verify the presence of amplification products of the correct size.

At least 40 clones from each leaf sample were tested with digestion using *MspI* restriction enzyme (Sibenzim, Russia). RFLP products were analyzed in 2% agarose gel. Tested clones had different rDNA PCR restriction patterns (Figure 2).

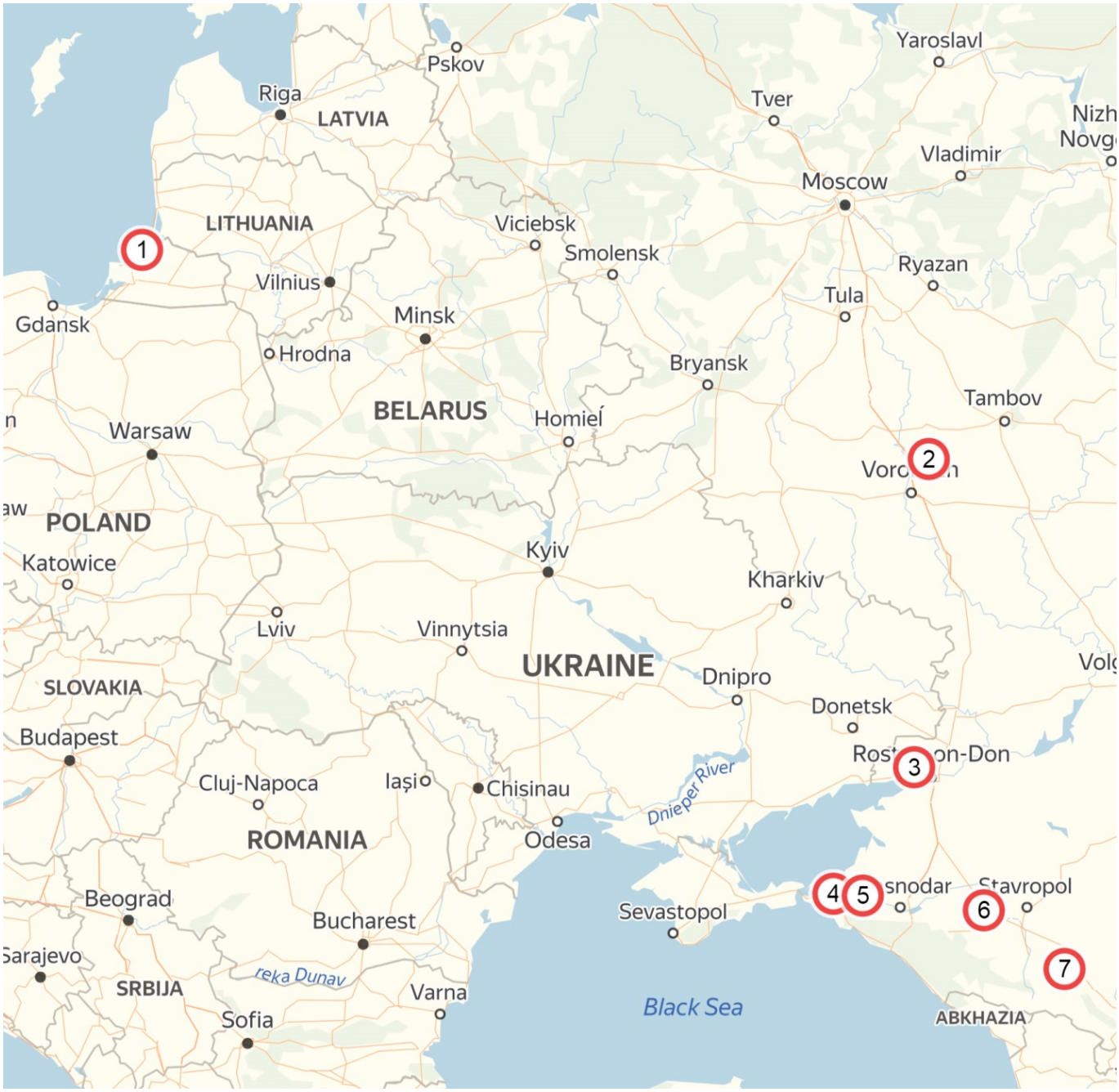
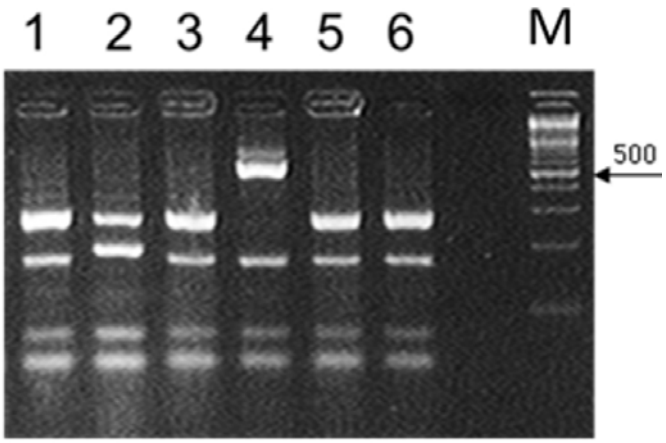


Figure 1. Collection sites (see Table 1)

For sequencing 20-30 clones with insertions (including clones with different restriction patterns) from every leaf sample were used. A total of 200 clones were sequenced. Sequencing reactions were performed following the BigDye terminator protocol (ABI Prism) with M13 primers. Sequences were analyzed by the Blast program at the Genbank (NCBI, USA) and at UNITE database. Both programs yielded identical results for almost all tested clones.

Analysis of fruits

In the field in Krasnodar Territory (site 5, Figure 1, Table 1), mycobiota of tomato fruits was analyzed by the method of isolation and analysis of axenic cultures. Green fruits with dark dry spots on the surface were used for tests. Fruits were washed carefully, and surface sterilized with 70% alcohol. Fruits slices were placed in moist chambers. Mycelium or spores were taken from alive



Lines1, 3, 5, 6: *Septoria lycopersici*; 2: *Podosphaera fusca*; 4: *Alternaria alternata*; M – 1 kb DNA Ladder

Figure 2. Restriction patterns of 700-bp fragment amplified by M13 primers and digested with *MspI*

tissue with a needle under the microscope and placed on a Petri dish with wort agar with the addition of penicillin. In total, 45 fruits were investigated. Isolation of fungal strains, DNA extraction, PCR amplification, extraction of amplicons, and sequencing were performed as described in Kutuzova et al. (2017). The species affiliation of tested isolates was determined by cultural and morphological characters and by sequences of species-specific DNA region ITS1-5,8S-ITS2 (primers ITS5 and ITS4, or ITS1F and ITS4) (White et al., 1990; Gardes and Bruns, 1993).

RESULTS

Analysis of fungal diversity in tomato leaves using sequencing of cloned PCR-amplified rDNA

In this study, a total of 14 unique rDNA sequences 482-682 bp long were obtained. After sequence analysis specific DNA regions of fourteen species were revealed in blighted leaves: *Septoria lycopersici* Speg., *Fulvia fulva* (Cooke) Cif. (=*Cladosporium fulvum*), *Didymella glomerata* (Corda) Qian Chen & L. Cai (=*Phoma glomerata*), *Cladosporium herbarum* (Pers.) Link, *Podosphaera fusca* (Fr.) U. Braun & Shishkoff, *Neocamarosporium goegapense* Crous & M.J. Wingf. (=*Phoma betae*), *Rhizoctonia solani* J.G. Kühn, *Candida albicans* (C.P. Robin) Berkhout, *Dioszegia hungarica* Zsolt, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Didymella lycopersici* Kleb., *Alternaria infectoria* E.G. Simmons, *Alternaria alternata* (Fr.) Keissl., *Cryptococcus tephrensis* Vishniac.

The most commonly occurring species were *A. alternata* (30% of all clones), *S. lycopersici* (9%), *F. fulva* (8%). *N. goegapense*, *R. solani*, *C. albicans*, *D. hungarica*, *C. cladosporioides*, *D. lycopersici*, *A. infectoria*, *C. tephrensis* were recovered with low frequencies. In the leaves from healthy plants without any visible symptoms DNA of three species was found: *Aspergillus versicolor* (Vuill.) Tirab., Annali, *A. alternata*, *Aureobasidium pullulans* (de Bary & Löwenthal) G. Arnaud.

The number of species per one sample (simple leaf) varied from two to six. The largest number of species was found in samples from Rostov region (sites 3 and 6, Table 1), four species were found in one sample from Kislovodsk (site 7), other samples contain DNA of 2-3 species (Table 1). Similar results were obtained after analysis of potato (*Solanum tuberosum*) and bitter-sweet nightshade (*Solanum dulcamara*) leaves (Kokaeva et al., 2018b, Kokaeva et al., 2019).

Analysis of tomato fruits mycobiota

Several fungal species were identified after the analysis of axenic cultures isolated from green diseased tomato fruits. *A. alternata* (GenBank accession number KU245685) was detected in most of the studied fruits. Other identified fungal species were *Phomopsis phaseoli* (Desm.) Sacc. (MH412692), *Alternaria solani* Sorauer (KY496637), *Fusarium equiseti* (Corda) Sacc. (MT588081), *Chaetomium cochliodes* Palliser (MT279444), *Clonostachys* sp. (MT588112), *Irpex lacteus* (Fr.) Fr. (MT276332), *Colletotrichum coccodes* (Wallr.) S. Hughes (MT292616).

DISCUSSION

South regions, including Krasnodar and Stavropol territories, Rostov region, are the main tomato producing areas in Russia. Development of fungal diseases cause a great crop loss. Appearance of new fungal species leads to decrease of efficiency of traditional protection schemes. Novel species of fungal pathogens can be resistant to traditionally applied plant protection products.

In this research both widespread on tomato and rare, not previously noted in Russia, fungal species were

Table 1. Identified fungal species in leaf samples using sequencing of cloned PCR-amplified rDNA

Indication on a map (Figure 1)	Collection site	Description of plants on the site	Identified species, GenBank accession numbers
1	Kaliningrad region. Kurshskaya kosa national park. Control plot with healthy plants.	Small private garden. Plants green. Leaves without any disease symptoms.	*Aspergillus versicolor* MT279494 *Alternaria alternata* KU245685 *Aureobasidium pullulans* MT279495
2	Voronezh region. Paninsky district.	Private garden. Plants were green and weakly affected. Leaves were green with dry necrotic lesions.	*Cladosporium herbarum* KU245683 *Rhizoctonia solani* KU245690 *Alternaria alternata* (KU245685)[1]
3	Rostov region, near Rostov-na-Donu city.	Commercial field. Strong affection of individual plants. Many ripen fruits had rot infection. Leaves were green with dry necroses. Sprinkler irrigation.	*Dioszegia hungarica* KU245691 *Cladosporium herbarum* (KU245683) *Neocamarosporium goegapense* KU245684 *Alternaria alternata* (KU245685) *Rhizoctonia solani* (KU245690) *Candida albicans* KU245692
4	The Krasnodar territory, Temryuk district.	Commercial field. Plants were green and weakly affected. Leaves were green with rare dry necrotic lesions. Drip irrigation.	*Fulvia fulva* KU245688 *Didymella lycopersici* MT279496
5	The Krasnodar territory, Slavyansk-na-Kubani district.	Commercial field. Strong affection of most plants and fruits. Leaves with many necrotic lesions. Sprinkler irrigation.	*Fulvia fulva* (KU245688) *Peyronellaea glomerata* KU245689 *Alternaria alternata* KU245685
6	Rostov region, Armavir district	Commercial field. Plants were weakly affected. Ripen fruits without any rots. Green leaves with dry necroses were collected. Sprinkler irrigation.	*Alternaria alternata* (KU245685) *Septoria lycopersici* KU245686 *Cladosporium herbarum* (KU245683) *Alternaria infectoria* MK131038 *Cryptococcus tephrensis* KU245693
7	Stavropol territory, Kislovodsk city.	Private garden 1. Plants were mainly green and weakly affected. Leaves were green with dry necrotic lesions.	*Septoria lycopersici* (KU245686) *Podosphaera fusca* KU245687 *Alternaria alternata* (KU245685) *Cladosporium cladosporioides* MK131039
7	Stavropol territory, Kislovodsk city.	Private garden 2. Plants were mainly green and weakly affected. Leaves were green with dry necrotic lesions.	*Septoria lycopersici* (KU245686) *Podosphaera fusca* (KU245687) *Alternaria alternata* (KU245685)

[1] sequence of the sample completely (100%) identical to submitted number in parenthesis

detected. *S. lycopersici*, *P. fulva*, *D. lycopersici*, *A. infectoria*, *A. alternata* — these ubiquitous pathogenic species occur on tomato all over the world. The pathogenicity of *A. alternata* in relation to different tomato varieties was studied in an earlier research (Kudryavtseva et al., 2017). Some identified species (*P. fusca*, *N. goegapense*, *P. glomerata*) are not typical for phytopathogenic mycobiota of tomato and were never observed in Russia. *P. fusca* is one of the causative agents of powdery mildew in cucurbits and some other angiosperm plants (Pérez-García et al., 2009). This species was never previously reported on tomato plants. However, this pathogen was found on Solanaceae plants: on *Nicotiana alata* in USSR (Braun, 1995), and on *Solanum macrocarpum* in Korea (Cho and Shin, 2004). *N. goegapense* (= *Phoma betae*) is a pathogen causing "zonal" leaf spot. The species was found in tomato plants in India (Mathur, 1979). *Peyronellaea glomerata* (= *Phoma glomerata*) have wide range of host plants. The pathogenicity of this microorganism was proved on potato tubers and leaves (Kranz, 1963). *P. glomerata* was detected on tomato in the Netherlands and potato in Germany (Aveskamp et al., 2010). This paper is, to available knowledge, the first report of *P. glomerata* and *N. goegapense* on tomato in Russia. *R. solani* is noted as the causative agent of foliar blight of tomato (Ivors et al., 2009), and it is noted that lesions are similar to the lesions of *A. alternata*. *R. solani* can inoculate stems and roots of *Solanum lycopersicum* (Pourmahdi and Taheri, 2015; Manning, 1980).

On a healthy leaf from control plot in Kaliningrad region (site 1) *A. pullulans*, a yeast-like saprophytic fungus, was found. It naturally inhabits plant and fruit surfaces. It was tested as a potential biocontrol agent against a wide range of pathogenic fungi. Italian researchers tried to use this fungus as a potential biocontrol agent against *Phytophthora infestans* on tomato (Francesco et al., 2017).

Investigation of tomato fruits revealed several widespread (*A. alternata*, *A. solani*, *C. coccodes*) and new tomato pathogens (*P. phaseoli*, *F. equiseti*, *I. lacteus*) and saprotrophic species *C. cochliodes* and *Clonostachys* sp. *A. alternata* was detected in most of the studied fruits. *C. coccodes* is a widespread tomato pathogen (Belov et al., 2018; Manning, 1980). *P. phaseoli* is known as a soybean pathogen; it was first discovered on tomato (Elansky et al., 2020). *F. equiseti* was found as a tomato pathogen in Asian countries (Akbar et al., 2018; Kamlesh and Ramchandra, 2017). *C. cochliodes* and *Clonostachys* sp. — saprotrophic soil fungi entering antagonistic relations with many soil microorganisms. The biocidal activity of *Clonostachys* against nematodes (Silva et al., 2015) and some fungal phytopathogens (Borges et al., 2015) was shown. Strains of *Chaetomium* are also used as biofungicide (Soytong et al., 2001). *I. lacteus* — basidiomycete, a white rot fungus, is not known as tomato pathogen.

In this research it was evaluated the growth ability of several strains on whole tomato fruits and its slices in a moist chamber. Symptomless, detached green tomato fruits, surface sterilized with ethanol (70%) and their slices were used for this test. According to our experiments, *Clonostachys* sp., *C. cochliodes*, *P. phaseoli*, and *I. lacteus* were not able to penetrate the tomato epidermis and infect fruits, but they developed well on fruits' cuttings. It seems that these fungi can parasitize on tomato fruits when a crack occurs on their surface. *F. equiseti* showed great aggressiveness, it can infect the tomato fruits through the epidermis (Chudinova et al., 2020).

A. solani and *C. coccodes* were not identified in cloning test, but present on the diseased fruits. In previous research with *Alternaria* spp. PCR identification (Kokaeva et al., 2018a) was found that 40% of leaf samples contain *A. solani* DNA in Temryuk (location 4, Figure1), 35% — in Kislovodsk (7), 7% in Rostov (6), 0% in Armavir (6). Our research with PCR identification of *C. coccodes* (Belov et al., 2018) revealed 54% of leaf samples with DNA of this fungus in Rostov (3), 28% in Slavyansk-na-Kubani (5), 4% in Armavir (6), 0% in Temryuk (4). In present research it was analyzed only one leaf sample per field; possibly, it was too small to find *C. coccodes* or *A. solani*. Microscopic exploration of large amount of tomato fruits in site 5 revealed these two pathogens. It shows that field research with sequencing of cloned PCR-amplified rDNA can be implemented for research of mycobiota and search for new fungal species on the plant, but not for screening for dangerous pathogens.

CONCLUSIONS

This research provides new information on the mycobiota of tomato leaves. Several new tomato pathogens were found. The emergence of new species shows the need for continuous improvement of disease control management and adjustments of the lists of plant protection products used.

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