J Plant Dis Prot (2018) 125:205–212
https://doi.org/10.1007/s41348-017-0135-3

ORIGINAL ARTICLE

Distribution of *Alternaria* species on blighted potato and tomato leaves in Russia

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Received: 30 March 2017 / Accepted: 13 October 2017 / Published online: 24 October 2017

Abstract *Alternaria* species are the causal agents of potato and tomato early blight disease. Three species-specific PCR primer sets were designed for *Alternaria alternata* sensu lato, *Alternaria solani* and *Alternaria infectoria* identification. The specificity of primers was confirmed by the absence of amplified products after PCR with DNA of *Alternaria* spp., other potato and tomato pathogenic fungal species and plant DNA. Constructed primers were applied for the survey of early blight agents on affected potato and tomato leaves collected in different regions of Russia. All three *Alternaria* pathogens were present in blighted leaves alone or in complex (*A. alternata* + *A. solani*, *A. infectoria* + *A. alternata* and *A. solani* + *A. infectoria*). *Alternaria alternata* (sensu lato) was the most abundant species. It was detected in 50% of tested potato leaves and 41% of tested tomato leaves (alone or in complex). *Alternaria solani* was found in 30 and 35% leaves, respectively. *Alternaria infectoria* occurred in potato and tomato samples occurred at a somewhat lower frequency (13 and 8%, respectively). Using the specific primers, it was possible to exclude the *Alternaria* disease in some leaves with symptoms similar to early blight.

Keywords Early blight · *Alternaria* sp. · Leaf blight · Potato diseases · Tomato diseases

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Introduction

Most *Alternaria* species are saprophytes commonly found in soil or on decaying plant tissues. Some species cause a large range of diseases affecting numerous economically important host plants, including cereals, potato, tomato, cabbage, broccoli, carrots, ornamental plants, citrus fruits and apples. The common symptoms of *Alternaria* diseases are necrotic lesions on leaves which are primarily concentric, often surrounded with yellow chlorotic tissue. This zone is created by the diffusion of fungal toxins (Saharan et al. 2016). While some of the *Alternaria* spp. infect potato leaves and tubers, affected plants still have properly functioning roots or vessels and keep providing water absorption or transport of nutrients.

In recent years with warm and dry summers, early blight became widespread in the central and southern part of Russia and in Europe and turned into one of most important diseases after late blight. Previous Russian studies postulated that the species *A. tomatophila* E.G. Simmons, *Alternaria solani* Sorauer, *A. alternata* (Fr.) Keissl., *A. tenuissima* (Kunze) Wiltshire, *A. infectoria* E.G. Simmons and *A. arborescens* E.G. Simmons are involved in the complex of pathogens causing "early blight" and "brown spot" on potato and tomato. In this report, we consider *A. tenuissima*, *A. arborescens* and *A. alternata* as one group, designated *A. alternata* sensu lato, because of minimal molecular variation between them (Woudenberg et al. 2015) and no significant differences in fungicide resistance (Pobedinskaya et al. 2012). In Brasil *A. grandis* E.G. Simmons has been published as causal agent of early blight too (Rodrigues et al. 2010). For verification of species from the complex causing early blight of potato and tomato in Russia, sequence analysis and morphological diagnostics of isolated pure cultures of *Alternaria* pathogens was done

previously. There were only 3 *Alternaria* species found in infected tissues: *A. alternata* (sensu lato), *A. solani* and *A. infectoria* (Orina et al. 2010; Elansky et al. 2012). *Alternaria tomatophila* or *A. grandis* has not earlier been found among tested leaf samples in Russia (Elansky et al. 2012).

While it is difficult to distinguish lesions caused by *A. solani* and *A. alternata* s.l., it is important to be able to diagnose which species is present due to their different resistances to fungicides, virulence on cultivars, optimal growth temperature (Pobedinskaya et al. 2012; Kapsa 2008; Landschoot et al. 2017; Tymon and Johnson 2014; Kudryavtzeva et al. 2017). Identification of the small-spored species causes problems especially. *Alternaria infectoria* is probably often confused with *A. alternata* because of their small conidia. Furthermore, isolates of *A. infectoria* and *A. alternata* produce different metabolites and toxins (Andersen and Thrane 1996). Conventional methods for identification of plant pathogens are time-consuming, since they require isolation of axenic cultures. In the case of *Alternaria* species, there is a need for fresh leaves with transportation arrangements to avoid contamination with secondary mycobiota including small-spore species of *Alternaria*.

In this study, we applied PCR technology and specially constructed species-specific primer sets for the survey of early blight agents (*A. solani*, *A. alternata* and *A. infectoria*) on affected potato and tomato leaves collected in different regions of European Russia.

Materials and methods

Fungal isolates

The following fungal isolates from the collection of mycology and algology department of the Lomonosov Moscow State University were used in this study: *Alternaria alternata*, *A. solani*, *A. infectoria*, *Phytophthora infestans* (Mont.) de Bary (strain P08LKL2/1), *Colletotrichum coccodes* (Wallr.) S. Hughes (strain C16MEPL3), *Helminthosporium solani* Durieu and Mont (strain H14M(Ch)PT1), *Rhizoctonia solani* J.G. Kuhn. (strain R13M2PT3), *Fusarium oxysporum* Schltdl. (strain F15MTL2), *Boeremia foveata* (Foister) Aveskamp, Gruyter and Verkley (= *Phoma exigua*) (strain Ph15MSdL4), *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (strain Cl15MPL1). Strains of *A. tomatophila* (MF-P208-011, CBS116704) and *A. avenicola* E.G. Simmons (MF-P067-011) were obtained from the laboratory of mycology and phytopathology of the All-Russian Plant Protection Research Institute. *Solanum tuberosum* L. (cv. Red Scarlett) and *S. lycopersicum* L. (cv. Dubrava) plants were grown in the greenhouses of the Lomonosov Moscow State University.

Sources of diseased samples

A total of 421 samples of potato and tomato leaves with early blight symptoms were collected at 17 sites in 9 regions of European Russia (Fig. 1, Tables 2, 3). Samples were collected annually in July from 2011 to 2015 by the authors themselves. Fresh leaves of potato and tomato were fixed in 70% alcohol solution or CTAB buffer immediately after sampling.

DNA extraction from fungal mycelia and plant tissues

DNA was extracted from a whole simple leaf lamina with one or multiple necroses by crushing samples in CTAB

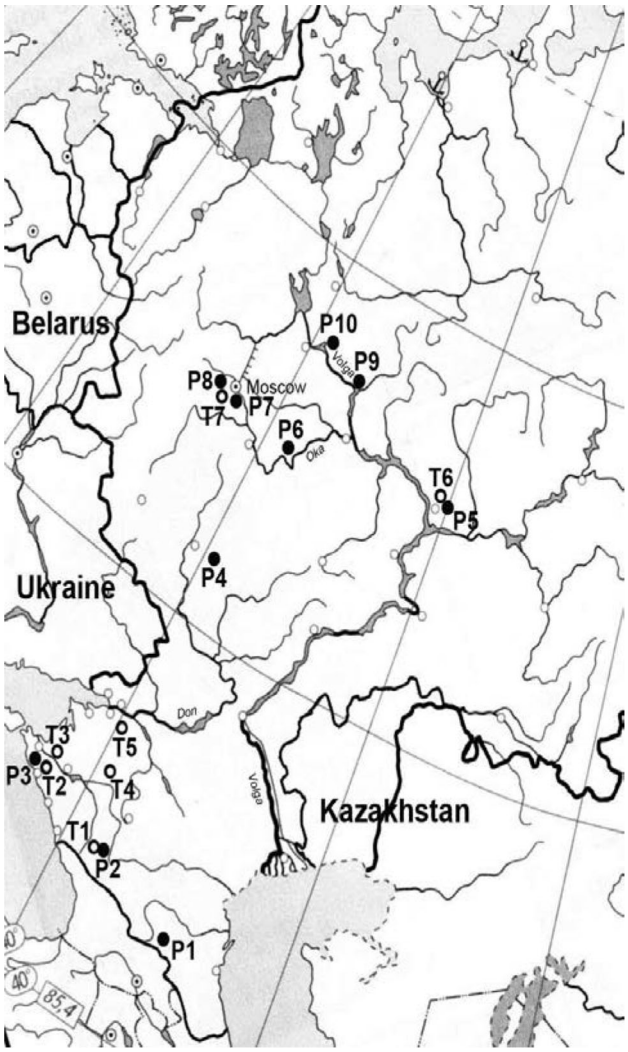

Fig. 1 Collection sites of infected potato (filled circle) and tomato (open circle) leaves (see Tables 2, 3)

DNA extraction buffer (0.5 M NaCl, 10 mM Tris–HCl [pH 7.5], 10 mM EDTA, 2% [w/v] CTAB) using liquid nitrogen as described by Griffith and Shaw 1998. Before DNA extraction mycelium of fungal isolates grew on pea-broth liquid nutrient media (125 g of frozen peas in 1 L of distilled water was boiled for 10 min, filtered through cheesecloth and autoclaved for 30 min at 1 atm). DNA concentration was determined using a spectrophotometer (NanoDrop 2000; Thermo Scientific, USA) by measuring absorbance at 260 nm. The final concentration of extracted DNA was adjusted to 50 ng/µl. All DNA samples were kept at − 20 °C.

PCR amplification

DNA amplifications were performed in 25-µl total volume containing 50 ng of DNA-template, 200 µM of deoxyribonucleotide triphosphate (dNTP), 0.2 µM of each primer (Table 1), 1.5 U of Taq polymerase (Promega Corp., Madison, WI) in the reaction buffer supplied by the manufacturer. As a negative control, 1 µl of purified water (milliQ) was used instead of fungal DNA. The amplification was performed on a Biometra T1 cycler (Biometra, Germany). DNA was denatured for 3 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at specific temperature (Table 1) for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. For sequence analysis primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC) (White et al. 1990) were used with annealing temperature 58 °C. After amplification, PCR products were electrophoresed on 1% agarose gel with ethidium bromide (0.5 µg/ml) in 0.5× Trisborate EDTA (TBE) buffer at constant power, 100 V, for approximately 1 h, and visualized and recorded with a UVP ImageStore 7500 UV Transilluminator (UVP Inc., Upland, CA). If needed, PCR product was isolated from the gel, cleaned using the "Cytokine" kit (Cytokine Co., Russia), and used for a sequence analysis.

DNA sequencing

PCR amplicons were sequenced using the BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and Applied Biosystems 3730 xl automated sequencer (Applied Biosystems, CA, USA). Each fragment was sequenced in both directions using the same primers described for PCR above. Contigs sequences were used to establish identity of the fungal isolates based on sequences similarity in GenBank using the BLASTn program for nucleotide–nucleotide analysis (version 2.0, National Center for Biotechnology Information, United States National Institutes of Health, Bethesda, MD, USA).

Nucleotide polymorphisms between tested species were identified and used for design of species-specific primers with Oligo software (Table 1). Blast software (GenBank) was used for primer specificity testing in silico.

Results

Design of specific PCR primers

Specific primers (Table 1) were designed on the nucleotide sequences of the ITS region of the rDNA gene from *Alternaria* axenic cultures, isolated from infected potato (leaves and tubers) and tomato (leaves and fruits) samples collected in different regions of Russia. Twenty four used isolates of *A. alternata* s.l. were from Moscow, Kostroma, Astrakhan, Leningrad regions, Mari El and Tatarstan republics, Caucasus; all sequences were similar to GenBank accession number KY524297. Seven isolates of *A. solani* were from the Astrakhan region and Far East of Russia (all sequences were the same as KY496637), the single strain of *A. infectoria* was isolated from the sample, collected in Kostroma region (KY496638).

Primer specificity and sensitivity tests using conventional PCR

The specificity of all primer sets was tested against genomic DNA from target Russian isolates of *Alternaria*, and

Table 1 PCR primers specific for *Alternaria* species

Primer	Annealing temperature	Size of product (bp)	Sequence 5′–3′	Specific for species
ITS5/MR	50 °C	505	GGAAGTAAAAGTCGTAACAAGG GACCTTTGCTGATAGAGAGTG	*A. alternata*
ITS5/SR	56 °C	460	GGAAGTAAAAGTCGTAACAAGG CTTGGGGCTGGAAGAGAGCGC	*A. solani*
Inf.pr/Inf.obr	56 °C	127	GACACCCCCCGCTGGGGCACTGC GGTTGGTCCTGAGGGCGGGCGA	*A. infectoria*

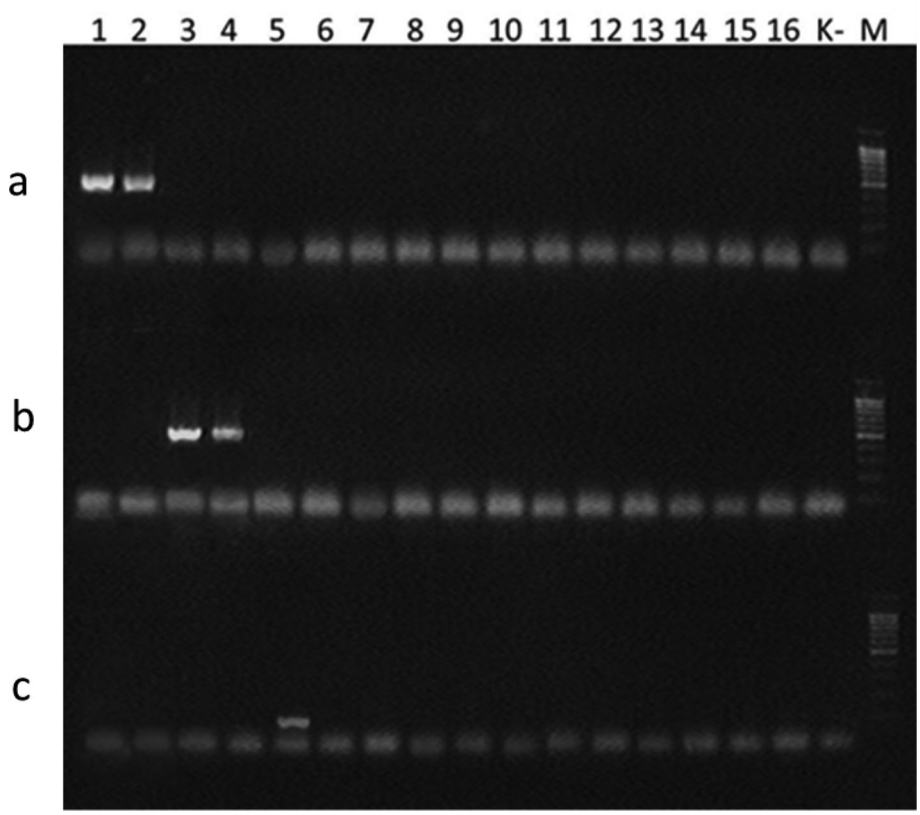

Fig. 2 Gel showing specificity of designed primer pairs: **a** ITS5-MR for *A. alternata*, **b** ITS5-SR for *A. solani*, **c** Inf.pr-Inf.obr for *A. infectoria*, but not from DNA of other pathogenic species. Lanes: 1, 2: *A. alternata* isolates, 3, 4: *A. solani* isolates, 5: *A. infectoria*, 6: *A. tomatophyla*, 7: *A. avenicola*, 8: *Phytophthora infestans*, 9: *C. coccodes*, 10: *Helminthosporium solani*, 11: *Rhizoctonia solani*, 12: *Fusarium oxysporum*, 13: *Boeremia foveata* (= *Phoma exigua*), 14: *Cladosporium cladosporioides*, 15: *Solanum tuberosum* L., 16: *S. lycopersicum* L., K-: negative control (dH_2O); M: DNA marker (100–1000 bp)

confirmed by the absence of PCR product after amplification of DNA from other *Alternaria* species or from a wide range of other fungal species pathogenic to solanaceous plants: *Phytophthora infestans*, *Colletotrichum coccodes*, *Helminthosporium solani*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Boeremia foveata*, *Cladosporium cladosporioides* (Fig. 2). Comparisons between primer sequences MR, SR, Inf.pr, Inf.obr with DNA database sequences (Blast tool) of other plant pathogenic fungi and bacteria revealed no significant levels of similarity.

The amplification sensitivity was confirmed with dilution series of DNA for target species. The minimum concentration of genomic DNA for amplification was 1 ng/µl for ITS5-MR (*A. alternata*), 100 pg/µl for ITS5-SR (*A. solani*) and 50 pg/µl for Inf.pr-inf.obr (*A. infectoria*) (Fig. 3).

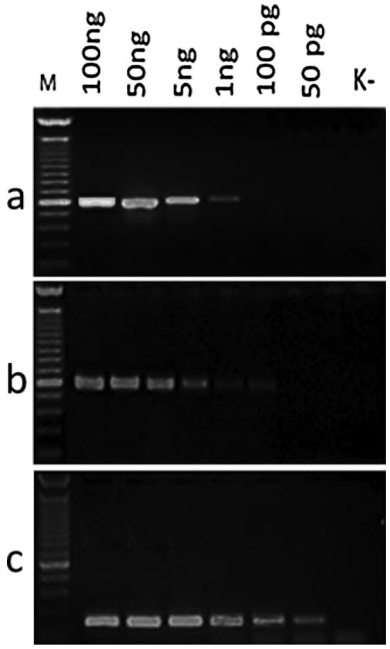

Fig. 3 Sensitivity test of specific primers: **a** amplification with ITS5-MR (*A. alternata*), **b** amplification with ITS5-SR (*A. solani*), **c** amplification with Inf.pr-inf.obr (*A. infectoria*). M-: DNA marker (100–1000 bp), K-: negative control (dH_2O)

Estimation of the distribution of the *Alternaria* species on blighted potato and tomato leaves in Russia

The obtained primers were used to test DNA from the infected potato and tomato leaves with necrosis or blight symptoms. An example of amplification results is shown in Fig. 4. All three target species *A. alternata* s.l., *A. solani* and *A. infectoria* were detected successfully in DNA samples from potato and tomato leaves (Tables 2, 3). It was noteworthy that *Alternaria* species were detected

simultaneously in samples. For example, positive signals of proposed PCR markers revealed coexistence of *A. alternata* and *A. solani* in samples 3, 4 and 6, and coexistence of *A. solani* and *A. infectoria* in sample 2 (Fig. 4.)

Alternaria species in potato leaves

Based on DNA analysis, *A. alternata* appeared as the most common species among the samples of potato leaves from 10 fields collected in 8 regions of Russia during 2011–2014. This species was detected in 50% of the samples and was present in all studied regions (Table 2). *Alternaria solani* was identified in 30% of DNA samples and was found in all studied regions except Krasnodar region. *Alternaria infectoria* was detected in 13% of samples and was not found in samples from Stavropol and Ryazan regions.

Simultaneous presences of two species in one host sample were considered separately, and the amount of these cases was equal to 16%. *Alternaria solani* and *A. alternata* co-occurrence was found in 9% of the samples and was detected in all regions except Ryazan region and the Krasnodar Territory. In 4% of the samples *A. alternata* and *A. infectoria* were present simultaneously, and only in 3% of the samples *A. solani* and *A. infectoria* were detected simultaneously. In 23% of the leaf samples with blight and necrosis symptoms, no agents of early blight were identified. None of the tested samples contained all 3 target *Alternaria* species simultaneously.

Alternaria species in tomato leaves

The outcome of surveying the tomato leaves was similar to potato sampling. In total, 115 leaf samples were analyzed from 7 fields of tomato in open soil from 5 regions of Russia (Table 3). In most cases (41%), tomato leaves contained *A. alternata* mycelium. However, *A. alternata* was not detected in samples from the Stavropol Territory. *Alternaria solani* was identified in 34% of DNA samples and was not detected in one investigated field in Rostov region. *Alternaria infectoria* was detected in 8% of the tested samples.

The simultaneous presence of species was identified in 11% of all examined samples, and the complex of *A. solani* + *A. alternata* was predominant (equal to 9%). The co-occurrence of *A. alternata* + *A. infectoria* and *A. solani* + *A. infectoria* was equal to 1% in each case. A larger number of tomato leaves (27%) with symptoms of blights had no agents of early blight identified (Tables 2, 3) in comparison with potato leaves.

None of tested samples of potato or tomato leaves has all 3 target *Alternaria* species simultaneously.

Discussion

Molecular methods are useful and fast tools for species identification. Several molecular methods for the detection and identification of phytopathogenic *Alternaria* species

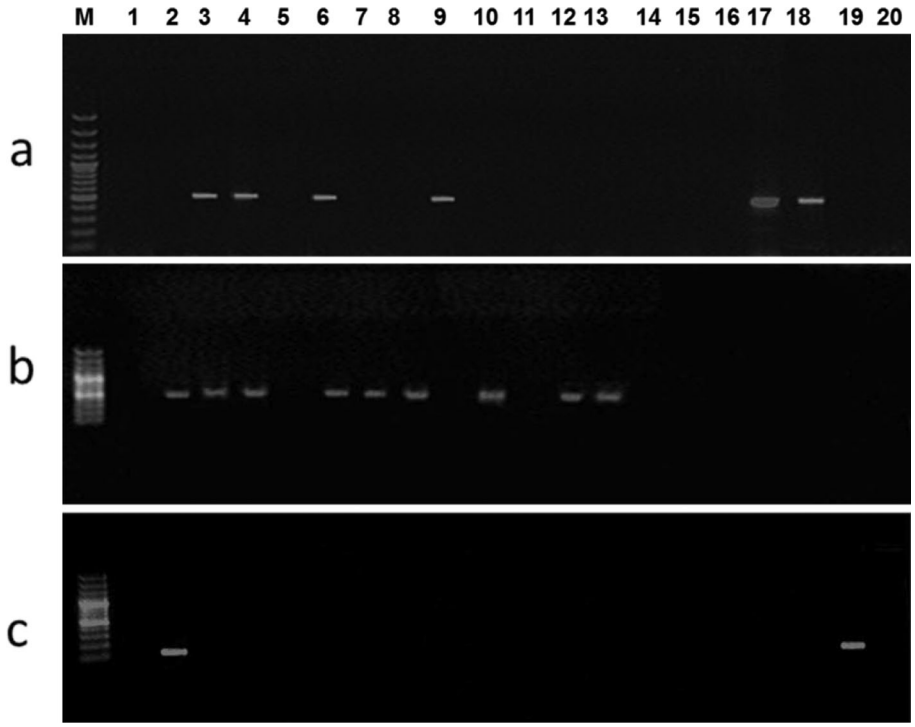

Fig. 4 Identification of *Alternaria* spp. in leaves using diagnostic primers. **a** amplification with ITS5-MR (*A. alternata*), **b** amplification with ITS5-SR (*A. solani*), **c** amplification with Inf.pr-Inf.obr (*A. infectoria*). M: DNA ladder 1 kb, 1–20: DNA samples of the tested leaves (part of the electropherogram)

Table 2 The occurrence of species of *Alternaria* on potato leaves from different regions of Russia

Place of collecting	Point on the map (Fig. 1)	Number of tested samples	Number of samples with *Alternaria* species						
			A.s.[a] only	*A.alt.* only	*A.inf.* only	*A.s.* + *A.inf.* together	*A.s.* + *A.alt.* together	*A.alt.* + *A.inf.* together	Not detected
Republic of North Ossetia-Alania, Vladikavkaz district	P1	8	0	1	0	0	5	2	0
Stavropol region, Kislovodsk city	P2	23	2	14	0	0	1	0	6
Krasnodar region, Anapa district	P3	8	0	4	2	0	0	2	0
Voronezh region, Panino district	P4	28	8	12	1	2	3	2	0
Tatarstan Republic, Kazan district	P5	78	10	25	2	0	3	0	38
Ryazan region, Kasimov district	P6	12	3	6	0	0	0	0	3
Moscow region, Lyubertsy district	P7	25	9	8	2	1	3	0	2
Moscow region, Odintsovo district	P8	67	21	17	5	2	9	1	12
Kostroma region, Makarovo district	P9	15	0	11	1	1	1	1	0
Kostroma region, Minskoe village	P10	42	3	17	4	2	2	4	10
Total		306	56	115	17	8	27	12	71
Total (%)			18	37	6	3	9	4	23

[a]*A.s.*, *A. solani*; *A.alt.*, *A. alternata* s.l.; *A.inf.*, *A. infectoria*

Table 3 The occurrence of species of *Alternaria* blight on tomato leaves from different regions of Russia

Place of collecting	Point on the map (Fig. 1)	Number of samples	Number of samples with *Alternaria* species						
			A.s. only	*A.alt.* only	*A.inf.* only	*A.s.* + *A.inf.* together	*A.s.* + *A.alt.* together	*A.alt.* + *A.inf.* together	Not detected
Stavropol region, Kislovodsk city	T1	17	6	0	0	0	0	0	11
Krasnodar region, Anapa district	T2	22	8	5	2	0	2	0	5
Krasnodar region, Temryuk district	T3	20	7	4	2	0	1	0	6
Rostov region, Armavir district	T4	13	0	6	2	1	0	1	3
Rostov region, Rostov district	T5	13	0	7	1	0	0	0	5
Tatarstan rep., Kazan district	T6	10	2	6	0	0	1	0	1
Moscow region, Odintsovo district	T7	20	6	8	0	0	6	0	0
Total		115	29	36	7	1	10	1	31
Total (%)			25	31	6	1	9	1	27

have been developed during the last decade. The Ain3F/Ain4R primer pair detects *A. infectoria* (Gannibal and Yli-Mattila 2007). A PCR test was proposed for the detection of *Alternaria* fungi in food products (Pavyn et al. 2011). An *Alternaria*-specific real-time PCR assay was used for quantitative estimation of *A. solani* and *A. alternata* presence in plant tissues during disease development (Leiminger et al. 2014).

In the present study, primer pairs ITS5/MR, ITS5/SR, Inf.pr/Inf.obr were designed based on the fungal ITS sequence. These primers successfully amplified the target DNA sequences of isolates from blighted potato and tomato samples (Fig. 2). They were specific enough to distinguish *A. alternata* s.l., *A. solani*, *A. infectoria* from other pathogenic *Alternaria* species (*A. tomatophila*, *A. avenicola*), or from other common plant pathogens of Solanaceae plants. The great advantage of the developed primers is the ability to detect low levels of target fungal DNA *in planta* material.

The presence of *Alternaria* spp. was investigated in 421 samples of diseased tomato and potato leaves, which includes 115 samples of tomato and 306 samples of potato. According to the literature, *A. solani* was indicated as the main causative agent of early blight of solanaceous crops (Dang et al. 2015). Nevertheless, in this study small-spored *A. alternata* (sensu lato) was found in a larger number of samples among potato and tomato blighted leaves. The large-spored species *A. solani* was predominant in Moscow region on potato and in the Krasnodar Territory on tomato. At the same time, it was not detected on tomato leaves in Rostov region and on potato in Anapa district of the Krasnodar Territory. The less frequent occurrence of *A. infectoria* may indicate that it is to a lesser extent involved in the disease development.

Apparently, all 3 species are widespread in the central and southern regions of Russia. This distribution can be explained by the inoculum migration with the seed tubers and the yield of potato and tomato tubers. Early potatoes and tomatoes are produced at these sites (P1–P3; T1–T25), and later transported from south to north. Potato seed tubers are mainly produced in the north and then distributed south. All appearances indicate that these factors influence the spread of the pathogens in the European part of Russia.

There is ongoing discussion about the importance of the *Alternaria* species in Early Blight. Some researchers are convinced that only *A. solani* is pathogenic (Turkensteen et al. 2010). In this case *A. alternata* would be a saprophyte, which colonizes leaf lesions caused by *A. solani* and is therefore a secondary invader. Nevertheless, the pathogenicity of *A. alternata* has been postulated also (Droby et al. 1984; Zheng et al. 2015). In our previous studies, it has been shown the ability of *A. alternata* to infect and the different virulence of *A. alternata* to potato and tomato cultivars (Kokaeva et al. 2015; Kudryavtzeva et al. 2017). Ardestani et al. (2010) demonstrated that *A. infectoria* could cause early blight symptoms on potato leaves in Iran. American researchers (Tymon et al. 2016) also isolated strains of *A. infectoria* group from potato leaves.

A considerable part of samples show the simultaneous presence of species and the complex of *A. solani* and *A. alternata* was predominant. Several studies have shown that *A. solani* and *A. alternata* could be isolated simultaneously out of EB typical symptoms (Babler et al. 2004; Latorse et al. 2010). Leiminger and Hausladen (2012, 2013) discuss the possibility that co-occurrence of *A. solani* and *A. alternata* could cause more severe infections.

Among the 23% of potato and 27% of tomato tested samples with early blight symptoms *Alternaria* pathogens were not detected. Thus, in P5 site *Alternaria* pathogens were not detected in almost half of tested leaves with early blight like symptoms. Possibly these symptoms were caused by other fungal pathogens. For example, *Colletotrichum coccodes* was found in leaves with early blight like symptoms collected in neighboring Mari El region (Kokaeva, Elansky, unpublished data). Another possible reason of necrosis can be environmental factors (Turkensteen et al. 2010).

Thus, primers designed can be used for specific amplification of molecular markers for *A. alternata*, *A. solani* and *A. infectoria* and provide the successful identification of these species even when the cultural tests are difficult because of morphological features.

Acknowledgements Financial support to the work with collected materials and fungal isolates was provided by the Russian Science Foundation (Project No. 14-50-00029). Ekaterina Skolotneva was supported by the State Budget Project (No. 0324-2016-0001); Lyudmila Kokaeva was supported by the Foundation for Assistance to Small Innovative Enterprises in Science and Technology (FASIE, Project No. 10566GU/2016).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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