PAGV – SPECIAL REPORT NO 18 – 2017, 181-190

Alternaria spp. and *Colletotrichum coccodes* in potato leaves with early blight symptoms

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ABSTRACT
Colletotrichum coccodes and *Alternaria* species are plant pathogenic fungi affecting different organs of potato, tomato, and some other plants. Species-specific primers were applied for the survey of *C. coccodes* and *Alternaria* spp. on affected potato leaves collected in different regions of Russia. All studied *Alternaria* pathogens were present in blighted leaves alone or in the combinations, such as *A. alternata* + *A. solani, A. infectoria* + *A. alternata, A. solani* + *A. infectoria*. *A. alternata* (*sensu lato*) was the most frequent species revealed in 50% of tested potato leaves (alone or in combination with other species). *A. solani* and *A. infectoria* were revealed in 30 and 13% of samples, respectively. The presence of a DNA region specific for *C. coccodes* was detected in DNA samples isolated from potato leaves collected in the Northern Ossetia, Kostroma region, and Mariy El Republic.

KEYWORDS
Colletotrichum coccodes, early blight, *Alternaria* spp.*,* leaf blight, potato diseases, anthracnose, black dot

INTRODUCTION

Alternaria species cause diseases of numerous economically important host plants, including potato, tomato, cereals, etc. Common symptoms of *Alternaria* infection are necrotic lesions on leaves, which are primarily concentric and are often surrounded with yellow chlorotic tissue. In recent years characterized by warm and dry summer seasons, early blight became widespread in the central and southern parts of Russia and in Europe and became one of most important diseases of potato being inferior to the late blight. Three *Alternaria* species were found in infected potato leaf tissues in Russia: *A. alternata* (in this report we consider *A. tenuissima*, *A. arborescens* and *A. alternata* as the same group, designated *A. alternata* sensu lato), *A. solani,* and *A. infectoria* (Orina et al., 2010, Gannibal, 2007, Elansky et al., 2012).
Colletotrichum coccodes (Wallr.) S. Hughes is a plant pathogenic fungus affecting different organs of tomato, potato, and a wide range of other plant species. In the case of potato tuber

infection, the fungus causes so-called black dot disease. This disease results in a peel exfoliation, significantly worsens the appearance of tubers, and causes water losses during storage. The black dot disease is observed in the majority of world potato-producing regions; the corresponding yield losses for susceptible cultivars may reach 30% (Johnson and Miliczky, 1993; Johnson 1994; Tsror et al., 1999).

Black dot development on leaves causes formation of necrotic lesions similar to early blight or brown spot symptoms (Johnson and Miliczky, 1993). According to US researchers, who collected leaves with early blight or brown spot symptoms (caused by *Alternaria solani* and *A. alternata*), in some years *C. coccodes* represented up to 5-10% of strains isolated from leaves (Tymon et al*.,* 2016). The development of the black dot disease on leaves and other above-ground parts of plants causes mass development of spores, which then infect other plant organs and other plants with drops of rain or irrigation water. The purpose of this study was the PCR-based investigation of *C. coccodes* and *Alternaria* sp. occurrence in potato leaves with early blight or brown spot symptoms collected in European Russia.

Another practically important issue is a differentiation between *A. alternata* s.l. and *A. solani* due to their different resistance to fungicides, virulence on cultivars, and optimal growth temperature (Pobedinskaya et al., 2012, Kapsa, 2008). In this study we applied PCR approach and species-specific primer sets for the survey of early blight agents (*A. solani*, *A. alternata* and *A. infectoria*) on affected potato leaves collected in different regions of European Russia.

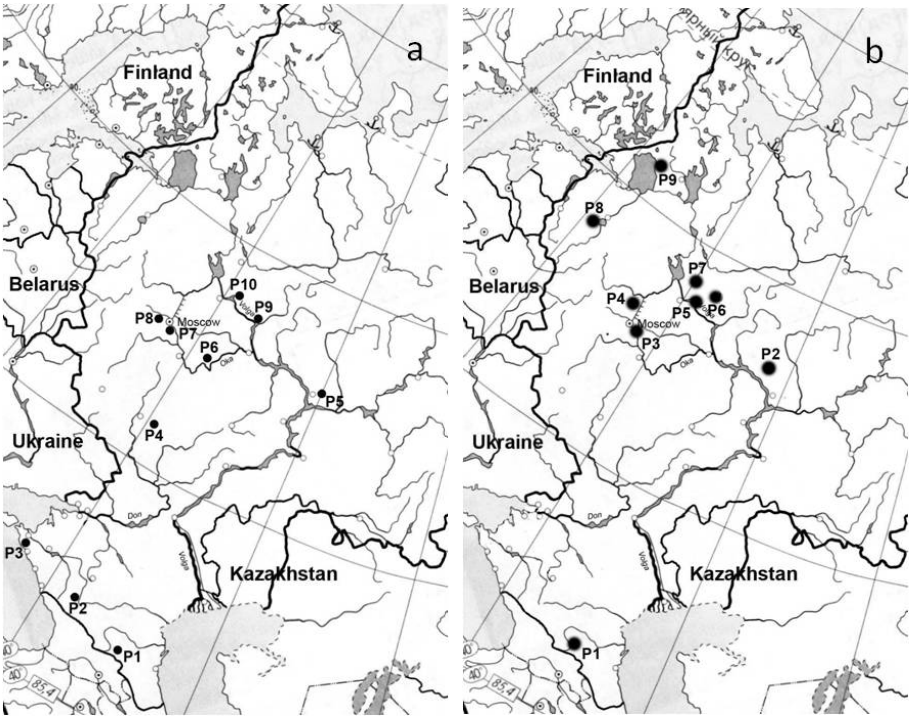

Figure 1. *Collection sites of infected potato leaves for: a) Alternaria tests (see Table 2), b) C. coccodes tests (see Table 3).*

MATERIALS AND METHODS

Sample collection. Potato leaves with clear manifestation of infection were collected from commercial fields and homestead plots in 17 sampling sites located in nine different regions of European Russia (Figure 1; Tables 2, 3). Samples were collected in August-September, when potato tubers reached a marketable size, but top parts of plants still remained green. During sampling, only one leaf per plant was collected, and the distance between the sampled plants was 5 m or more. For each sampling site, 20-25 green and non-wilted leaves with clear dark necroses, similar to early blight lesions, were collected. The leaves were immediately put into 70% ethanol to prevent the development of secondary mycobiota on dead tissues.

DNA extraction. DNA was extracted from the whole simple leaflets with one or multiple necroses by crushing samples in CTAB 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 Kutuzova et al., 2017. DNA concentration was determined spectrophotometrically at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The final concentration of extracted DNA was adjusted to 50 ng/µl. All DNA samples were stored at –20°C.

PCR amplification. Isolated DNA was first amplified using ITS1F and ITS4 primers (Table 1), which selectively amplify DNA of the most of ascomycetes and basidiomycetes. Only samples with successful PCR reaction were used in the further work. A selective amplification at the next stage of the study was provided by the use of species-specific primers (Table 1, Figure 2).

The total PCR reaction volume was 25 µl. Reaction mixture contained 1 µl of DNA template, dNTP (200 µM), primers (0.2 µM of each, Table 1), 1.5 U of Taq polymerase and reaction buffer (Promega Corp., Madison, WI). In the case of negative control, 1 µl of milliQ water was used instead of fungal DNA. Amplification was performed using a Biometra T1 cycler (Biometra, Germany). Thermal cycling conditions included an initial denaturation step at 94°C for 3 min followed by 30 cycles of denaturation (94°C for 30 s), 30-s annealing at specific temperature (Table 1), elongation (72°C for 45 s), and a final elongation stage (72°C for 5 min). PCR products were electrophoretically separated on 1% agarose gel supplemented with ethidium bromide (0.5 µg/ml) in a 0.5× TBE buffer at 100 V for approximately 1 h, then visualized and recorded using an ImageStore 7500 UV transilluminator (UVP Inc., Upland, CA).

Table 1. *List of primers used in the study*

Primer	Nucleotide sequence	Annealing temperature, °C	Specific for species	Reference
ITS4/ITS1F	5'-TCCTCCGCTTATTGATATGC 5'-CTTGGTCATTTAGAGGAAGTAA	54°C	Fungi	White et al. 1990 Gardes and Bruns 1993
Cc1NF1/ Cc2NR1	5'-TGCCGCCTGCGGACCCCCCT 5'-GGCTCCGAGAGGGTCCGCCA	66°C	*C. coccodes*	Cullen et al. 2002
ITS5/MR	5'-GGAAGTAAAAGTCGTAACAAGG 5'-GACCTTTGCTGATAGAGAGTG	50°C	*A. alternata*	Kokaeva et al., 2017
ITS5/SR	5'-GGAAGTAAAAGTCGTAACAAGG 5'-CTTGGGGCTGGAAGAGAGCGC	56°C	*A. solani*	
Inf.pr/ Inf.obr	5'-GACACCCCCCGCTGGGGCACTGC 5'-GGTTGGTCCTGAGGGCGGGCGA	56°C	*A. infectoria*	

RESULTS

Our study showed the occurrence of *C. coccodes* and *Alternaria* species in green potato leaves with dry necrotic lesions. *A. alternata* appeared to be the most common species revealed in samples from 10 fields located in eight different regions of Russia. This species was found in 50% of samples and was present in all studied regions (Table 2). *A. solani* was identified in 30% of samples and was found in all studied regions except the Krasnodar region. *A. infectoria* was detected in 13% of samples and did not present in samples from the Stavropol and Ryazan regions.

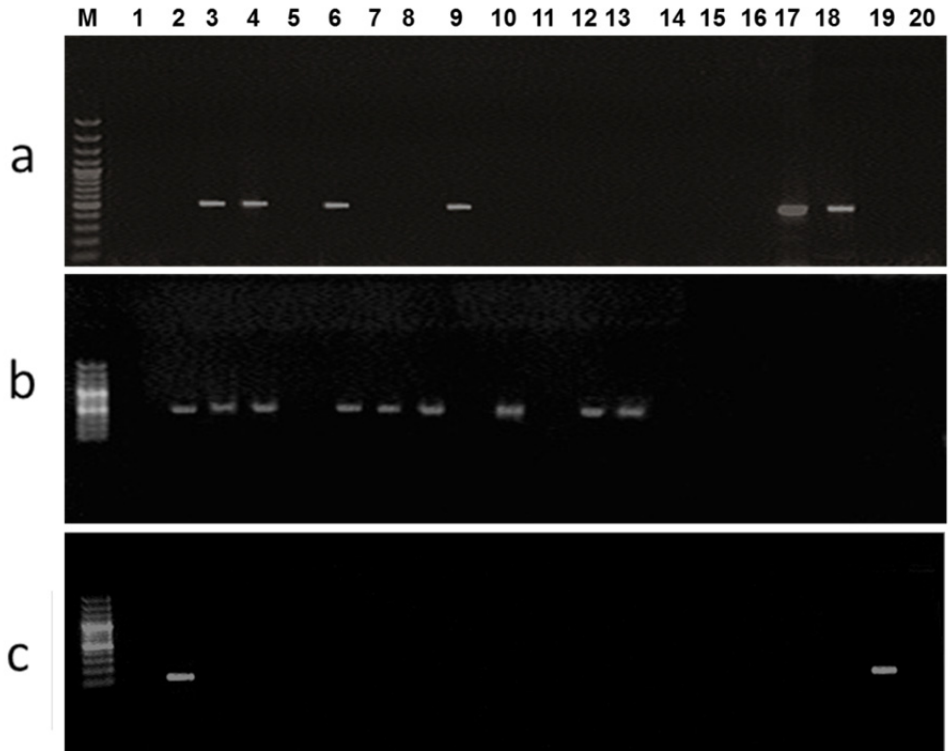

Figure 2. *Identification of Alternaria spp. using species-specific primers. a - amplification with ITS5-MR (A. alternata), b - amplification with ITS5-SR (A. solani), c - amplification with Inf.pr-Inf.obr (A. infectoria) M – DNA ladder 1kb., 1 – negative control, 2-20 - DNA samples of tested leaves.*

A simultaneous presence of two species in one host sample was considered separately (16% of samples). The co-occurrence of *A. solani* and *A. alternata* was revealed in 9% of samples from all regions except the Ryazan and Krasnodar regions. The combination of *A. alternata* and *A. infectoria* was revealed in 4% of samples. The co-occurrence of *A. solani* and *A. infectoria* was detected only in 3% of samples. Finally, no early blight agents were found in 23% of samples with blight and necrotic symptoms. None of the tested samples contained all 3 target *Alternaria* species.

C. coccodes was revealed in five samples from the North Ossetia, Kostroma region, and Mariy El Republic (Table 3). In the North Ossetia, plants of the sampled field were green and actively vegetated; only single leaves with dry necroses were observed. In the Mariy El Republic and Kostroma region (Strelnikovo), samples were collected at the end of the vegetation season; plants were strongly affected with early and late blights and the haulm started to wither.

DISCUSSION

The presence of *Alternaria* spp. was examined in 306 potato samples. According to the data of other authors, *A. solani* is 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. Large-spored *A. solani* prevailed on potato leaves collected in the Moscow region, but was not detected on potato in the Anapa district of the Krasnodar region.

There is an ongoing discussion about the importance of *Alternaria* species for the early blight disease. Some researchers are convinced that only *A. solani* is pathogenic (Turkensteen et al., 2010). In this case, *A. alternata* may be a saprophyte, which colonizes leaf lesions caused by *A. solani,* and, therefore, represents a secondary infection. Nevertheless, some authors postulated the pathogenicity of *A. alternata* (Droby et al., 1984, Zheng et al., 2015). In our previous studies we showed the ability of *A. alternata* to infect potato and tomato leaves and the different virulence of *A. alternata* to various cultivars (Kokaeva et al., 2015, Kudryavtseva et al., 2017). This paper confirms that *A. alternata, A. solani,* or *A. infectoria* alone are capable to induce the disease. The possibility of *A. infectoria* to cause early blight on potato leaves was also shown in Iran (Ardestani et al., 2010). Some American researchers (Tymon et al., 2016) also isolated strains of the *A. infectoria* group from potato leaves.

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

Region	Sampling site (Fig.1a)	Number of samples analyzed[a]	Number of samples containing DNA of:						
			A.s.[b] *only*	*A.alt. only*	*A.inf only.*	*A.s.+ A.inf.* together	*A.s. + A.alt.* together	*A.alt. + A.inf.* together	No *Alternaria* DNA
Republic of North Osetia-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
Ryazan region, Kasimov district	P5	12	3	6	0	0	0	0	3
Tatarstan republic, Kazan district	P6	78	10	25	2	0	3	0	38
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] *Number of samples with positive results for the ITS 1F and ITS4 primers.*

[b] *A.s. – A. solani, A.alt – A. alternata s.l., A.inf. – A. infectoria.*

A considerable part of samples demonstrated a simultaneous presence of the studied species with the prevalence of the complex of *A. solani* and *A. alternata*. Several studies showed that *A. solani* and *A. alternata* could be isolated simultaneously from the lesions with typical EB symptoms (Bäßler et al., 2004; Latorse et al., 2010). In some of these studies, a high pathogenicity of the *A. solani – A. alternata* complex is discussed (Leiminger and Hausladen 2012, 2013). *Alternaria* pathogens were not detected in 23% of tested potato samples with clear early blight symptoms; probably, these symptoms were caused by other fungal pathogens, such as *Cladosporium* sp., *Colletotrichum coccodes,* etc.

Table 3. *Occurrence of C. coccodes DNA in the collected samples potato leaves*

Sampling site (Figure 1b)	Regions	Number of samples analyzed[a]	Number of samples containing DNA of *C. coccodes*[b]
P1	North Ossetia, Mikhailovskoe village	10	1
P2	Mariy El Republic, Yoshkar-Ola city	12	3
P3	Moscow region, Lyubertsy district	24	0
P4	Moscow region, Dmitrov district, Rogachevo village	3	0
P5	Kostroma region, Kostroma district, Strel'nikovo village	12	1
P6	Kostroma region, Susanino district	3	0
P7	Vologda region, Gryazovets district, Rostilovo village	12	0
P8	Novgorod region, coast of Il'men lake	9	0
P9	Karelia, Lyaskela village	11	0
	Total (potato)	**96**	**5**

[a] *Number of samples with positive results for the ITS 1F and ITS4 primers.*

[b] *Number of samples with positive results for the Cc1NF1and Cc2NR1 primers.*

The performed study demonstrated the occurrence of *C. coccodes* on potato leaves collected in different regions of European Russia. The affection of leaves and above-ground stem parts of potato by *C. coccodes* was observed after the artificial inoculation (cuticle injury by a sand blaster with the subsequent incubation under high-moisture conditions), and was followed by high yield losses (Nitzan et al., 2006; Johnson, 1994, Mohan et al., 1992). Such injury of live green leaves caused formation of necrotic lesions similar to early blight symptoms, but without concentric rings. The edges of necroses were often surrounded by the yellowing, and the further disease development resulted in a leaf wilt (Johnson and Miliczky, 1993). Aggressive isolates were able to infect intact leaves (Andrivon et al., 1998). During harvesting, spores may migrate from leaves to damaged tubers and infect them. Therefore, potato tuber protection requires application of effective systemic or translaminar fungicides on senescent haulm. The chemical desiccation of top parts of potato plants before harvesting may be also helpful.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 15-29-02512).

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