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STUDIES ON THE MYCOBIOTA OF BLIGHTED *SOLANUM DULCAMARA* LEAVES

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Mycobiota of affected bittersweet nightshade (*Solanum dulcamara*) leaves has been studied. The leaves were collected on the territory of Lomonosov Moscow State University (Vorobyovy gory, Moscow). Sampled leaves were instantly frozen at –75°C for DNA extraction or placed immediately into moist chambers for isolation of fungal cultures. DNA was extracted from the whole leaf. Ribosomal DNA (including ITS1 and ITS2 regions) gene banks were constructed in *Escherichia coli* by cloning PCR products generated with primer pairs ITS1f and ITS4. Fragments from the cloned inserts were sequenced and compared to known rDNA sequences. Sixteen fungal species were revealed by cloning and isolation of fungal cultures: *Alternaria alternata*, *Aureobasidium pullulans*, *Boeremia exigua* (= *Phoma exigua*), *Botrytis cinerea*, *Cladosporium cladosporioides*, *C. herbarum*, *C. tenuissimum*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, *Coniothyrium fuckelii*, *Fusarium oxysporum*, *Phoma herbarum*, *Phytophthora infestans*, *Thanatephorus cucumeris*, *Thielavia basicola*. Four taxa were identified at the level of genera (*Cryptococcus* sp., *Mycosphaerella* sp., *Phialophora* sp., *Phoma* sp.). All collected leaf samples contained from 5 to 8 different fungal taxa. Most of the found species were known pathogens of solanaceous plants. Some species could produce toxins or cause human allergies.

Key words: bittersweet nightshade, mycobiota of leaves, plant pathogenic fungi, *Solanum dulcamara*, species identification

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Wild *Solanum* species inhabit urban waste lands and roadsides and may be found close to potato and tomato plantations. Sometimes *Solanum* species are used as ornamentals for urban and suburban gardening; however, they can act as alternate hosts for pathogens affecting solanaceous crops (potato, tomato, eggplant, and other). Bittersweet nightshade (*Solanum dulcamara* L.), which is a perennial liana, belongs to the most wide-spread wild *Solanaceae* species of Central Russia. *S. dulcamara* plants may provide a natural late blight depositary (Cooke et al., 2002; Flier et al., 2003).

S. dulcamara plants with late blight lesions have already been found on the territory of the Moscow State University. The pathogenicity and spore production of *Phytophthora infestans* (Mont.) de Bary isolates collected from this plant were determined using artificially infected potato and tomato leaves (Elansky et al., 2015). Bittersweet nightshade could also serve as a host plant for the overwintering of potato virus M (Perry and McLane 2011), tomato Pepino mosaic virus (Stobbs, Greig, 2014) and potato psyllid *Bactericera cockerelli*, as a vector for bacteria *Candidatus liberibacter solanacearum*, which caused the "Zebra chip" disease (Murphy et al., 2013). In addition, several potato and tomato pathogenic fungi were also reported for *Solanum dulcamara* plants: *Boeremia lycopersici* (Cooke) Aveskamp, Gruyter et Verkley (= *Ascochyta lycopersici* Brunaud) in USA (Anonymus, 1960), *B. exigua* (Desm.) Aveskamp, Gruyter et Verkley (= *Phoma exigua* var. *exigua* Desm.) in Poland (Mułenko et al., 2008), *Phytophthora infestans* (Elansky et al., 2015; Deahl et al. 2010), and *Verticillium dahliae* Kleb. in Canada (Ginns, 1986).

Spores of many fungal species affecting wild solanaceous plants (e.g., *Cladosporium* spp. and *Alternaria* spp.) may provoke allergic reactions in humans. An increased concentration of airborne spores may lead to various disorders of sensibilized persons (Gabriel et al., 2016; Sindt et al., 2016).

The purpose of this study was the investigation of mycobiota associated with live *Solanum dulcamara* leaves. To improve the identification of fungal species from affected leaf samples, two approaches were used: the isolation of fungi followed by axenic culture analysis and the cloning of species-specific DNA regions in *Escherichia coli* and further clone library sequencing.

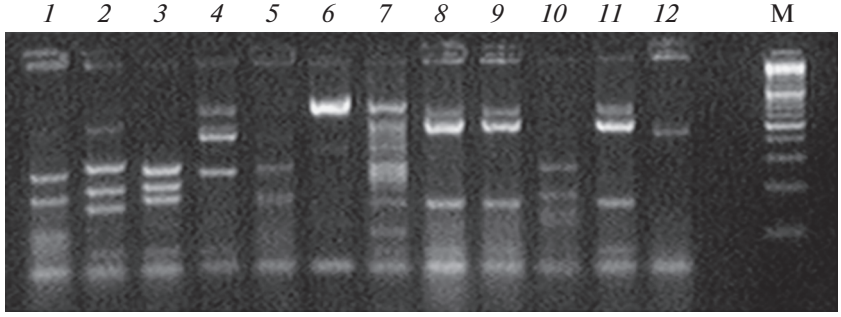

Fig. 1. Electrophoregram of the restriction analysis of clones produced from the sample 4: 1 – *Cladosporium herbarum*, 2 – *Thielavia basicola*, 3 – *Colletotrichum acutatum*, 4 – *Phoma herbarum*, 5, 10 – *Mycosphaerella* sp., 6 – *Aureobasidium pullulans*, 8, 9, 11 – *Alternaria alternata*, 12 – unidentified *Pezizomycotina*. M – DNA marker (100–1000 bp).

MATERIALS AND METHODS

Solanum dulcamara leaves with visible affection symptoms were collected in 2015 and 2017 (August – September) on the territory of the Lomonosov Moscow State University (Vorobyovy gory). The sampling area (Botanical garden) represented an upland surrounded by multi story houses and located within 7 km from the center of Moscow city. The nearest potato and tomato plantations were located 17 km away from the sampling area, small kitchen gardens – 10 km away.

Twenty leaves with necrotic lesions were sampled from several plants. Sixteen leaves were instantly placed into moist chambers for the isolation of fungi and axenic culture analysis and four leaves were frozen at –75°C for molecular genetic analysis.

Each leaf sample was placed upside down into a moist chamber consisting of a Petri dish with moistened filter paper. After 2–3 days of incubation at 22–25°C, leaves were microscoped. Fungal conidia, sclerotia or mycelia fragments were removed using a fine sterile dissecting needle and transferred to the center of a Petri dish with oatmeal agar supplemented with benzylpenicillin sodium salt (1000 U/ml) to prevent bacterial growth. Cultures were incubated at 22°C until the diameter of a fungal colony reached 25–30 mm. Then a mycelia fragment from the edge of colony was collected with a sterile needle and placed into a fresh Petri dish with an agar medium. The obtained fungal strains were stored in tubes with slant agar at 5°C. Fungal species were identified based on a morphology and micromorphology of cultures or and by sequencing of species-specific genome regions. To analyze axenic fungal cultures by sequencing, ITS5 and ITS4 primers were used according to White et al. (1990). Morphological features of fungal species were compared with the descriptions given in handbooks (Simmons, 2007; Seifert et al., 2011) or with the data from the Q-bank Fungi database (http://www.q-bank.eu/Fungi/).

Leaflets were obtained from the same plants from Botanical Garden. Leaflets were placed on moist chambers, adaxial side up. Leaflets remained unwounded. For sporulating species 3 spore suspensions (10 000 spores/ml) drops of 10µl each were placed per leaflet. For non-sporulating species leaves were infected by mycelium from axenic cultures. Negative controls were drops of sterile distillated water. Petri dishes were kept at room temperature (22°C) for 7 days for disease development (14 h daylight). Lesion development and size were assessed at this point. The experiment was carried out in three replicates.

Total DNA was extracted from each of affected leaves according to the standard CTAB protocol (Kutuzova et al 2017). Amplified rDNA fragments included a part of the *18S* gene region, internal transcribed spacer ITS1, *5.8S* gene, internal transcribed spacer ITS2, and part of a *28S* gene region. Universal ITS1f и ITS4 primers (CTTGGTCATTTAGAGGAAGTAA/TCCTCCGCTTATTGATATGC) were used (White et al., 1990; Gardes, Bruns, 1993). PCR was carried out using commercial PCR core kits (Laboratoriya Izogen Ltd, Moscow, Russia). The following PCR conditions were used: one cycle for 3 min at 96°C, 30 cycles with 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C, and 3 min at 72°C. The electrophoretical separation of PCR products was carried out in a TBE buffer and standard 1.5% agarose supplemented with 0.5 µg/ml ethidium bromide as a fluorescent tag. The gels were visualized under a UV light. Amplicons of the required length were extracted from gels using a CleanUp kit (Evrogen Ltd, Moscow, Russia), then inserted into a pAL-TA vector (Evrogen Ltd.) and used for the transformation of *Escherichia coli* (Dh5a strain) cells, in accordance to Inoue et al. (1990). The resulting clone library was examined by restriction analysis of the amplified DNA insertion using MspI restriction endonuclease (Fig. 1). Based on the obtained results, different restriction insertion profiles were selected.

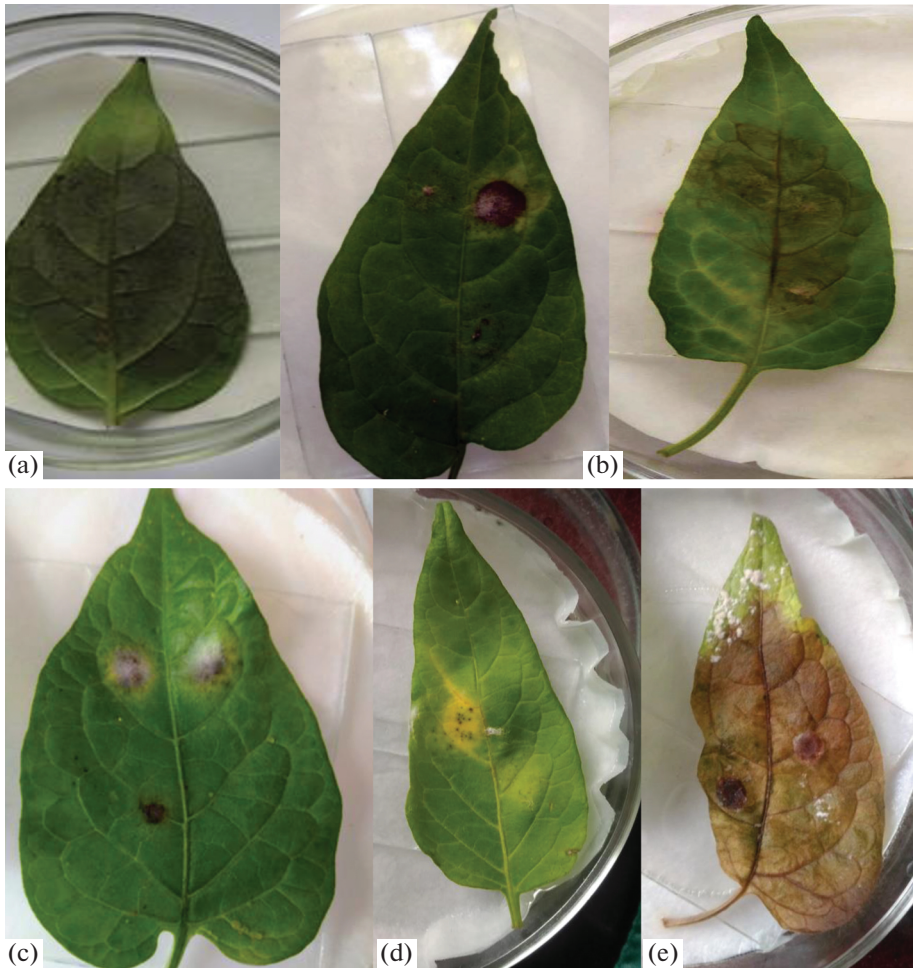

Fig. 2. Detached leaf assay. Lesions caused by *Phytophthora infestans* (a), *Botrytis cinerea* (b), *Alternaria alternata* (c), *Epicoccum purpurascens* (d), *Thanatephorus cucumeris* (e).

The plasmid DNA was extracted from a sample selected according to the Lee and Rasheed protocol (1990), and nucleotide insertions were sequenced.

Sequencing was carried out by the Evrogen company (Moscow, Russia). Plasmid DNA was sequenced using a BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and an Applied Biosystems 3730 × l automatic sequencer (Applied Biosystems, CA, USA). The resulted nucleotide sequences were used for the species identification by a comparison with the GenBank databases using BLASTn software.

RESULTS

Oomycete and fungal species isolated from the leaves of bittersweet nightshade by a culture dependent method included *Alternaria alternata* (Fr.) Keissl, *Boeremia exigua*, *Botrytis cinerea* Pers., *Coniothyrium fuckelii* Sacc., *Epicoccum purpurascens* Ehrenb., *Fusarium oxysporum* Schltdl., *Phytophthora infestans*, *Thanatephorus cucumeris* (A.B. Frank) Donk.

Lesions appeared on leaflets inoculated with *Phytophthora infestans*, *Botrytis cinerea*, *Alternaria alternata*, *Epicoccum purpurascens*, *Rhizoctonia solani* (Fig. 2). Of the 3 strains of *Boeremia exigua*, only one strain caused the appearance of chlorotic spots. First infection symptoms (necrosis) was observed on the third day after inoculation. *Coniothyrium fuckelii*, *Cladosporium* sp., *Fusarium oxysporum* don't create lesions.

The following 14 fungal and oomycete taxa were revealed via the cloning and further sequencing of DNA isolated from bittersweet nightshade leaves: *Alternaria alternata* (Genbank accession number KU366278), *Aureobasidium pullulans* (de Bary) G. Arnaud (KU366277), *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (KU366279), *Cladosporium herbarum* (Pers.) Link (KU366276), *C. tenuissimum* Cooke (KU182495), *Colletotrichum acutatum* J.H. Simmonds

Table 1. Fungal and oomycete species revealed in bittersweet nightshade leaf samples by a cloning procedure

Leaf sample, No	Number of clones obtained	Number of different restriction profiles	Fungal and oomycete taxa detected
1	11	6	*Colletotrichum gloeosporioides*, *Cladosporium tenuissimum*, *Cryptococcus* sp., *Phialophora* sp., unidentified *Pezizomycotina*
2	14	5	*Alternaria alternata*, *Cladosporium cladosporoides*, *Cryptococcus* sp., *Phoma destructiva*, *Phytophthora infestans*
3	18	6	*Cladosporium cladosporioides*, *Cladosporium herbarum*, *Cryptococcus* sp., *Phoma* sp., *Thielavia basicola*, unidentified *Pezizomycotina*
4	13	8	*Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium herbarum*, *Colletotrichum acutatum*, *Mycosphaerella* sp., *Phoma herbarum*, *Thielavia basicola*, unidentified *Pezizomycotina*

(KU366280), *C. gloeosporioides* (Penz.) Penz. et Sacc., *Cryptococcus* sp., *Mycosphaerella* sp., *Phialophora* sp., *Phoma herbarum* Westend. (KU366282), *Phoma* sp., *Phytophthora infestans, Thielavia basicola* Zopf (KU366283). *Alternaria alternata* and *Phytophthora infestans* were revealed by both culture-dependent and DNA-based techniques.

We have failed to identify 7 clones up to the species level. The closest similarity with the NCBI database reference sequences was found for the *Pezizomycotina* subdivision. However, nucleotide sequences within these clones were different. The phylogenetic analysis allowed us to split them in two distinct groups, one included six clones with similar sequences and another was represented by only one clone.

Each leaf sample analyzed by a cloning procedure hosted a number of fungal and/or oomycete taxa, which often co-occurred within the same infection lesion (Table 1).

DISCUSSION

According to the authors' knowledge, this was the first complex study of mycobiota inhabiting the leaves of *Solanum dulcamara* plants growing far from fields of agricultural solanaceous crops. Using cloning (4 leaf samples) and culture-dependent techniques (16 samples), we revealed 18 fungal taxa. Among the species revealed, *Boeremia exigua*, *Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum* and *Thanatephorus cucumeris* were known to be typical pathogens of solanaceous plants, which infect potato and tomato plants in Russia. Some of the identified fungal taxa were not typical for these crops or have not been observed in Russia yet. *Coniothyrium fuckelii* was revealed on potato plants in Poland (Mułenko et al., 2008). Yeast-like *Aureobasidium pullulans* is known as epiphyte or endophyte of a wide range of plant species (Andrews et al., 2002). It was reported as a tomato pathogen in Brazil and USA (Mendes et al., 1998) and also was isolated from potato leaves with clear blotch symptoms in the Leningrad Region of Russia (Gannibal, 2007).

Colletotrichum acutatum had a worldwide distribution and was pathogenic for a wide range of crops. The species was reported for such host plants as *Solanum lycopersicum* (Vichova et al., 2012), *Capsicum annuum* (Jelev et al., 2008), and *S. betaceum* (Jones, Perez, 2012). *Colletotrichum gloeosporioides* was also known to be a common plant pathogen found on both leaves (McKenzie, 2013) and fruits (Weir et al., 2012) of *Solanum melongena*. *Phialophora* species, which could be either pathogens or saprotrophs (Barnett, Hunter, 1972), were observed on solanaceous crops. For example, *P. richardsiae* (Nannf.) Conant and *P. parasitica* Ajello, Georg et C.J.K. Wang were revealed on tomato plants in Poland (Mułenko et al., 2008) and on potato plants in Greece (Thanassoulopoulos, Giapanoglou 1994), respectively. *Thielavia basicola* was a fungus pathogenic for more than 200 plant species (Shew, Meyer, 1992). It was found on tomato plants in Italy (Venturella, 1991) and has possibly represented the main cause for tobacco stem and root rot in China (Tai, 1979).

A number of plant pathogenic fungi might be harmful not only for plants but also for animal and human health. According to some data, several fungal species revealed during our work (*Alternaria alternata*, *Aureobasidium pullulans*, species of the genera *Cladosporium*, *Cryptococcus*, *Mycosphaerella*, and *Phoma)*, might provoke allergic reactions (Adkinson et al., 2013). *Alter-*

naria species were able to produce some toxins (altertoxin I, tentoxin, tenuazonic acid) and mutagenic compounds (alternariols) dangerous for humans (Scott et al., 2012).

The co-occurrence of several fungal species in a single affected leaf was noteworthy. Each of the four examined leaf samples harbored 5–8 different fungal species, i.e., complex infections predominated in the affected plant tissues. Some other researchers also reported the same phenomenon. Even low concentration of *Alternaria tenuissima* inoculum, accompanied by *A. solani* conidia, promoted the affection of plant tissues (Orina, 2011). There was also data confirming the pathogenicity of *A. solani* combined with *A. alternata* (Leiminger, Hausladen, 2011). Associations of *A. tenuissima*, *A. dumosa*, *A. arborescens*, *A. infectoria* and *A. interrupta* were observed and examined in Iran (Ardestani et al., 2010). A high pathogenicity of microorganism complexes was also reported for other host plants. Simultaneous co-inoculation of legume plants with leaf-attacking fungal species *Mycosphaerella pinodes* (Berk. et A. Bloxam) Vestergr. and *Phoma medicaginis* var. *pinodella* (L.K. Jones) Boerema impeded the disease progress and reproduction of the fungi (Le May et al., 2009). However, a consecutive inoculation with these pathogens significantly increased the intensity of the disease. Co-occurrence of several fungal species on a single leaf or even within the same necrotic spot did not necessary mean that an "all-at-once" colonization occurred. The infection could be started by a single biotrophic species and then subsequently accompanied by other hemibiotrophic or necrotrophic species.

Thus, 18 different fungal and oomycete taxa were revealed on *Solanum dulcamara* leaves, and most of them were pathogens of *Solanaceae* plants. Some of the species revealed have not been earlier reported as the pathogens of solanaceous plants in Russia, but were reported in other countries. In addition, some fungi provoking allergic reactions in immunocompromised humans were identified among the revealed species.

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Микобиота пораженных листьев *Solanum dulcamara*

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Изучена микобиота пораженных листьев паслена сладко-горького (*Solanum dulcamara*), произрастающего в Ботаническом саду Московского государственного университета им. М.В. Ломоносова

(Воробьевы горы, Москва). Исследование проводилось двумя методами: выделение чистых культур из пораженных листьев и клонирование ПЦР продуктов рДНК (ITS1–5.8S–ITS2) в *Escherichia coli*. Фрагменты из клонированных вставок секвенировали и сравнивали с известными последовательностями (GenBank – NCBI). Всего было обнаружено шестнадцать видов грибов: *Alternaria alternata*, *Aureobasidium pullulans*, *Boeremia exigua* (= *Phoma exigua*), *Botrytis cinerea*, *Cladosporium cladosporioides*, *C. herbarum*, *C. tenuissimum*, *Colletotrichum acutatum*, *C. gloeosporioides*, *Coniothyrium fuckelii*, *Fusarium oxysporum*, *Phoma herbarum*, *Phytophthora infestans*, *Thanatephorus cucumeris*, *Thielavia basicola*. Все исследованные образцы ДНК отдельных листьев содержали от 5 до 8 различных видов грибов.

Ключевые слова: микобиота листьев, паслен сладко-горький, фитопатогенные грибы, *Solanum dulcamara*