Detection and identification of "black foot" pathogens of grapes in Crimea

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Abstract. At the present stage of development, stable economic efficiency of viticulture is possible if a decrease in the viability of grape plants, accompanied by a decrease in their productivity and lifecycle reduction, is prevented. Progressive grapevine weakening can be caused by both climate change and affection of perennial wood by the complexes of phytopathogenic fungi. In the last two decades, in all major grape-cultivating states, the root rot or "black foot" of grapes has been attributed to the most harmful diseases of perennial wood, especially in nurseries and young plantations. Presently, 30 fungal species are known to be associated with this disease, with the most common genera being *Campylocarpon, Cylindrocladiella, Dactylonectria, Ilyonectria, Neonectria, Pleiocarpon, and Thelonectria*. The article presents the results of laboratory researches and field experiments aimed at studying the etiology of inhibition of grape plant development in the conditions of Crimea. For the period from 2017 to 2021, in the vineyards of Mountain-Valley, South-Western and Central-Steppe Crimea, the affection of bushes of grape varieties 'Moldova', 'Aligote', 'Arcadia', 'Lora', 'Rkatsiteli' with the disease "black foot" or root rot of grapes was diagnosed for the first time. Using of molecular biological approach based on the polymerase chain reaction (PCR) made it possible to establish that "black foot" pathogens in Crimean vineyards include *Dactylonectria macrodidyma* (Halleen, Schroers & Crous) L. Lombard & Crous, 2014 and *Ilyonectria destructans* (Zinssm.) Rossman, L. Lombard & Crous, 2015.

1 Introduction

Modern viticulture all over the world is facing the phenomenon of constant and progressive decline in the viability of grape plants, accompanied by a decrease in the productivity of grapevine, an increase in the intensity of its damage by various diseases, as well as early or sudden loss. In addition to climate change, the progressive grapevine weakening can be caused by damage to perennial wood by the complexes of phytopathogenic fungi,

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responsible for such diseases as esca, eutypa dieback, escoriosis, botryosphaeria dieback and root rot “black foot” of grapes. These diseases affect cropping capacity of grapes, quality of wine and plant life in almost all major countries involved in the cultivation of grapes. Global financial losses are estimated at more than 1.5 billion US dollars per year [1, 2].

Root rot or "black foot" of grapes is currently considered to be one of the most significant diseases of perennial wood. For the first time this disease was registered in 1961 in France, and in the last two decades its distribution has been recorded in industrial plantations and nurseries in many viticultural regions of the world, including Australia, Brazil, USA (California), Canada, China, France, Iran, Italy, New Zealand, Portugal, South Africa, Spain, Switzerland, Turkey, Uruguay, etc. [2-10]. First of all, root rot or "black foot" of grapes affects young grape plants under the age of eight years [4]. The most common symptoms associated with "black foot" of grapes are: reduced root biomass, formation of root cankers (necrotic lesions), blackening under the bark, and necrosis of xylem tissue in the basal part of the rootstock. Affected grape plants are characterized by weak growth vigor, formation of defective shoots with fanleaves, small chlorotic leaves with marginal and interveinal necrosis of leaf blade, and delayed or absent flowering [3–8].

Currently, 30 species of fungi are known to be associated with root rot or "black foot" of grapes, with the most common genera being *Campylocarpon, Cylindrocladiella, Dactylonectria, Ilyonectria, Neonectria, Pleiocarpon* and *Thelonectria*, and species - *I. liriodendri* and *D. macrodidyma* [2, 4-10]. In 2013-2015, such species as *Dactylonectria torresensis, Ilyonectria liriodendri* and *Thelonectria blackeriella* were isolated from young grape plants and rootstocks with symptoms of "black foot" in the vineyards and nurseries in Italy [5]. A three-year survey (2013-2015) of five grape nurseries carried out in the Western Cape region (South Africa) showed that the predominant “black foot” pathogens isolated from grapevines were *Campylocarpon fasciculare, Ca. pseudofasciculare* and *Dactylonectria macrodidyma* [6]. In 2014, in vineyards of Hormozgan province (Iran), 25 isolates, collected from the injured plants, were identified as *Campylocarpon fasciculare* (14), *Fusarium solani* (7) and *F. decemcellulare* (4) [7]. In 2015, in a year after planting, during the survey of a young vineyard (‘Sauvignon Blanc’ on SO4 rootstock) in Lednice (South Moravia, Czech Republic), about 30% of plants showed characteristic symptoms of “black foot” disease: necrotic lesions of roots, a decrease in their growth, and necrosis of wood at the base of the rootstock. The isolates collected were identified as *Dactylonectria torresensis* [8]. In 2016-2018, when surveying 15 nurseries in Northern Spain, 11 species, which belong to the genera *Dactylonectria, Ilyonectria, Neonectria* and *Thelonectria,* were identified, including *Dactylonectria alcacerensis, D. macrodidyma, D. novozelandica, D. pauciseptata, D. torresensis, Ilyonectria liriodendri, I. pseudodestructans, I. robusta, Neonectria quercicola, Neonectria sp. 1* and *Thelonectria olida* [9]. In 2017-2019, field studies carried out in Chinese provinces Guangxi, Hebei, Ningxia, Shanxi and Xinjiang found that the number of plants with “black foot” symptoms in the surveyed vineyards varied from 0.1% to 1%. From the samples of plants with disease symptoms, we have obtained 50 fungal isolates, which, based on morphological and multigenic phylogenetic analysis, were identified as *Cylindrocladiella lageniformis*, *Dactylonectria torresensis*, *D. macrodidyma*, *D. alcacerensis* and *Ne-onectria sp.1*. Among these five species, *D. macrodidyma* turned out to be the most aggressive [2]. Micromycetes are the pathogens of "black foot", mainly attributed to soil saprotrophs. They develop on dead residues of both woody and herbaceous plants. The infection remains in soil for several years as a mycelium or resting spores (chlamydospores). Grape nursery can be a source of infection. In case of planting healthy grape seedlings into infected soil, it is quite possible that they become infected through the root system or injuries on the lower part of the trunk with poor callus

formation. The infection can also occur through the injuries of aerial parts of the plant, in this case it spreads down. The infection can also be transmitted by moving of contaminated soils and soil (drainage) waters [2].

Currently, the search and development of effective protective and control measures aimed at preventing the loss of grape plants, damaged by pathogens of wood diseases, is relevant.

The goal of the research is to study the etiology of inhibition of grape plant development in the vineyards of Crimea, species diagnostics of "black foot" pathogen of grapes, including the use of molecular genetic methods.

2 Study objects and methods

Field studies were carried out in 2017-2021 in grape plantations of 15 enterprises in four main viticultural zones of Crimea: South-Coastal (SCC), South-Western (SWC), Mountain-Valley (MVC) and Central-Steppe (CSC). Route surveys, recordings and observations were timed to coincide with main phenological stages of grape development in plantations of wine and table varieties typical for the areas of study. Diagnostics of species to identify causative agents of systemic grape diseases (wood diseases) were carried out in Plant Protection Laboratory of the FSBSI Institute Magarach of the RAS and Biological Laboratory of the STC JSC Schelkovo Agrokhim.

The studies were carried out in accordance with methodological approaches generally accepted in national and international practices and adapted to grape agrocenoses. To identify fungi that cause grape diseases and to study their morphological and culture-based properties, the procedures adopted in microbiological practice were used: setting up in a humid chamber, inoculation on nutrient media. Fungi were identified using manual guides, databases and publications [5-7, 9, 11].

3 Results and discussion

As a result of the research, carried out in 2017-2021, when conducting route surveys in plantations of typical wine and table varieties of main viticultural zones of Crimea, cases of depressed development or loss of grape plants were recorded: in the vineyards of the Mountain-Valley Crimea in the plots of ‘Moldova’ and ‘Aligote’ varieties in 2017; in the South-Western Crimea in the vineyards of ‘Arcadia’ and ‘Lora’ varieties in 2018, and ‘Rkatsiteli’ in 2019; in the Central-Steppe zone of viticulture in the plot of ‘Rkatsiteli’ variety in 2021.

Depressed condition of grape plants was observed in the studied plots of table and wine varieties ‘Moldova’ and ‘Aligote’ of one of the enterprises situated in the Mountain-Valley Crimea. Annual shoots were mainly characterized as defective as had a pronounced fanleaf. On average, according to the inspection results of grape bushes by rows in different plot points, it was found that up to 68% of shoots on 76% of bushes were defective. A detailed examination of cross-sections of various parts of grape bushes, in general, showed the destruction of xylem tissues. Depending on the age, damage degree to conductive tissues varied: for a perennial trunk above the place of grafting - necrosis occupied 95% and 100% of cross-sectional surface; for a trunk below the grafting place - necrosis was total; for a 3-4-year-old grapevine - necrosis in the form of spots and sectors was reaching 70% of the cross-sectional surface; for annual shoots - both normal condition and presence of necrosis up to 30% of cross-sectional surface in the form of sectors were registered. As a result of

excavations carried out in the plot of 'Moldova' variety, presence of necrotic damage to main roots of grape bushes was revealed.

Isolation of fungal structures - macroconidia, microconidia, chlamydospores from necrotic tissue sectors of 'Moldova' and 'Aligote' perennial wood samples, their microscopic examination and determination of morphometric characteristics; obtaining, resulting from cultivation on potato-dextrose agar (PDA), of white colonies, as well as from dark orange to browns with dry consistency, made it possible to assume, with a high degree of probability, the affection of grape plants from the surveyed areas by the pathogen "black foot" or root rot of grapes - one of species of the genus *Cylindrocarpon* and/or *Cylindrocladiella*.

In the vineyards of table grape varieties 'Lora' and 'Arcadia', enterprises of the South-Western Crimea were observing the loss of 15-37.5% of bushes. On average, according to the results of grapevine inspection by rows in different places of the plot, it was found that: up to 100% of shoots on 70-80% of bushes were defective with chlorotic leaves; up to 10-30% of bushes suffered from drying of one vine arm. A detailed cross-sectional examination of various parts of grape bushes revealed the destruction of xylem, presence of circular or sectorial necrosis on the wood: for perennial trunk above the place of grafting, the area of necrosis was 70% of cross-sectional surface; for a 4-5-year-old grapevine, the area of wood necrosis was reaching 50-70% of cross-sectional surface; for a 3-4-year-old grapevine - circular necrosis of conductive tissues, necrosis in the form of sectors - up to 30% of cross-sectional surface.

Based on morphological and morphometric characteristics of fungal structures, and pure culture on PDA, presence of pathogens of "black foot" or root rot of grapes, presumably, by species of *Cylindrocarpon* genus, was diagnosed on wood samples taken from the plot of 'Lora' and 'Arcadia' varieties: on roots - *Dactylonectria torrensensis* Wollenw, Petit et al; on perennial woods - *Cylindrocarpon cylindroides* Wollenw.

Similar symptoms (weak bush growth vigor, formation of defective shoots with fanleaves, chlorotic leaves with marginal and interveinal necrosis, formation of root cankers, necrosis of xylem tissue) were observed when examining plots of 'Rkatsiteli' variety at two enterprises in the South-Western and Central-Steppe Crimea in 2019 and 2021, respectively. Isolates of *Cylindrocarpon* spp. were collected from perennial wood.

Thus, basing on the nature of symptoms causing damage to grape plants, and study results of fungal structure morphometric parameters isolated from necrotic sectors of perennial wood tissues, as well as the colonies obtained on PDA, the disease "black foot" or root rot of grapes was diagnosed with pathogens being species of genera *Cylindrocarpon* and/or *Cylindrocladiella, Dactylonectria* (Fig. 1).

Fig. 1. Seven-day mycelium of *Dactylonectria macrodidyma* grown on PDA at 25°C.

Reliable specific identification of *Cylindrocarpon* spp. according to the morphometric (phenotypic) features is difficult. Therefore, a molecular-biological approach based on the polymerase chain reaction (PCR) was used for specific diagnostics. The method's principle consists in multiplying the quantity of specific DNA, typical only for the target micromycete resulting from enzymatic polymerization reaction. Primers, flanking the species-specific genome sectors, were synthesized at Sintol LLC (Moscow) according to the recommendations from new literature sources [13–15]. Thus, sets of reagents for identification of the following pathogens of grapevine wood diseases were obtained: *Eutypa lata, Diaporthe ampelina, Phyllosticta ampelicida, Fomitiporella mediterranea, Phaeoacremonium spp., Phaeoacremonium minimum, Phaeomoniella chlamydospora, Agrobacterium vitis, Cylindrocarpon liriodendra, Cylindrocarpon macrodidymum, Cylindrocarpon pauciseptatum* and *Ilyonectria radicicola (*synonym *Cylindrocarpon destructans).*

Complete genomic DNA was extracted from mycelium according to the protocol of Lee & Taylor, 1988 [16]. Cell walls of the seven-day-old fungal mycelium were destructed by grinding in a pounder. Then, 2% CTAB extraction buffer was added, and after incubation at 65°C, a purification using mixture of phenol : chloroform : isoamyl alcohol (25:24:1) and further precipitation with isopropanol were carried out. DNA was dissolved in 50 µl of TE-buffer. The amount and purity of resulting DNA was determined spectrophotometrically. As a result, a DNA preparation of acceptable quality was obtained in an amount sufficient for PCR.

Polymerase chain reaction was carried out on a four-channel programmable amplifier TP4-PCR-01-"Tertsik" (LLC DNK-Tekhnologiya, Moscow). Two isolates of *Cylindrocarpon* spp. from 'Rkatsiteli' grapevine of South-Western (2018) and Central-Steppe (2021) viticulture zones of Crimea were identified in the work. To control the specificity of PCR-test, the DNA of *Alternaria alternata* and *Botrytis cinerea* was used, on the genome of which the selected primers should not be annealed. Visualization of the results was carried out electrophoretically.

On the isolate of DNA from the Central-Steppe viticultural zone, a single specific fragment of approx. 390 bp was obtained, amplified with primers to *Dactylonectria macrodidyma* (Halleen, Schroers & Crous) L. Lombard & Crous, 2014. Homotypic genbank synonym: *Cylindrocarpon macrodidymum*, *Ilyonectria macrodidyma* (Halleen, Schroers & Crous) P. Chaverri & C. Salgado. Basionym: *Neonectria macrodidima* (Halleen, Schroers & Crous, 2004).

The species-specific site of internal transcribed spacer (ITS) was chosen as a target to construct these primers: Mac1 5'-CCCTGATTACATTTAAGAAGT-3' and MaPa2 5'-TGATYCGAGGTCAAACG-3 [17]. The genome sector flanked by this pair of primers amounted to 387 bp. (Fig. 2).

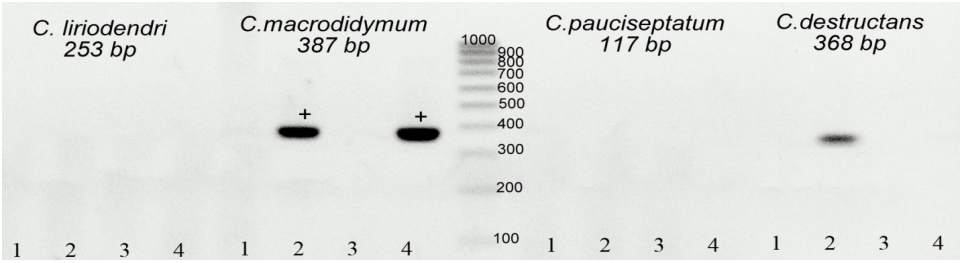

Fig. 2. Identification results for *Cylindrocarpon* spp. in PCR with primers to *C. liriodendra*, *D. macrodidyma*, *C. pauciseptatum* and *C. destructans*. The specific fragment is amplified with primers to *Dactylonectria macrodidyma* (387 bp) on isolates from 'Rkatsiteli' grapevine in the South-Western Crimea (track 2) and 'Rkatsiteli' from the Central-Steppe Crimea (track 4). A product specific to *C. destructans* is amplified on 'Rkatsiteli' from the Central-Steppe Crimea (track 4). Tracks 1 and 3 show the results of PCR with *Alternaria alternata* and *Botrytis cinerea*.

Grapevine from the South-Western Crimea was infected with a complex of pathogens consisting of *Dactylonectria macrodidyma* (Halleen, Schroers & Crous) L. Lombard & Crous, 2014 and *Ilyonectria destructans* (Zinssm.) Rossman, L. Lombard & Crous, 2015, basionym: *Ramularia destructans* (Zinssm., 1918); homotypic synonym: *Cylindrocarpon destructans*; heterotypic synonym: *Ilyonectria radicicola* (Gerlach & L. Nilsson) P. Chaverri & C. Salgado 2011, basionym: *Nectria radicicola* (Gerlach & L. Nilsson, 1963). Product size 368 bp, amplifies with primers Destruc-2F 5'-GTGCCTGYTTCGGCAGC-3' and Destruc-2R 5'-CTGTTTYCCAGTGCGAGGTGTGC-3' [15].

Primers for identifying other species of fungi associated with grapevine wood diseases did not generate any PCR product (data not shown).

As a result of diagnosing species using a molecular biological approach based on polymerase chain reaction, it was found that "black foot" pathogens in the vineyards of Crimea included the species *Dactylonectria macrodidyma* (Halleen, Schroers & Crous) L. Lombard & Crous, 2014 and *Ilyonectria destructans* (Zinssm.) Rossman, L. Lombard & Crous, 2015. The developed technique can be used for specific diagnostics of fungi as causative agents of grape wood diseases, isolated into pure culture or from the affected grapevines.

4 Conclusion

As a result of the studies provided, the etiology of inhibition of grape plant development (weak growth vigor of bushes, formation of defective shoots with fanleaves, chlorotic leaves with marginal and interveinal leaf necrosis, formation of root cankers, necrosis of xylem tissue) in the vineyards of Mountain-Valley, South-Western and Central-Steppe Crimea was established. Based on the nature of symptoms of the affected grape plant, and study results of morphometric parameters of fungal structures, isolated from necrotic sectors of perennial wood tissues, as well as the colonies obtained on PDA, the disease "black foot" or root rot of grapes was diagnosed in Crimea for the first time, with pathogens being species of genera *Cylindrocarpon* and/or *Cylindrocladiella, Dactylonectria*. Using of molecular-biological approach based on polymerase chain reaction made it possible to establish that *Dactylonectria macrodidyma* (Halleen, Schroers & Crous) L. Lombard & Crous, 2014 and *Ilyonectria destructans* (Zinssm.) Rossman, L. Lombard & Crous, 2015 belong to the pathogens of "black foot" in the vineyards of Crimea.

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