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The mycological study of conifers in Tbilisi and its surroundings

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Abstract: Extensive microbiological research was carried out in Tbilisi (capital of Georgia) and its surroundings to determine the causes of massive disease and dieback of urban coniferous plantations. The biological material was picked up in June–July 2020 from trees with various degrees of the disease in 42 different localities. 247 conifers (15 species) were examined microbiologically. 1 169 samples of microscopic fungi were isolated. Based on cultural-morphological and molecular-genetic (PCR) studies, 34 strains were identified to the species level, 17 to the genus level, and 1 strain to the family level. Ascomycota were represented by 15 families and 33 species, Basidiomycota by 1 family and 2 species and Zygomycota by 2 families and 2 species. Among the isolated strains, 9 species were clearly dominant and found in all studied coniferous species: *Alternaria alternata* (Fr.) Keissl, *Sphaeropsis sapinea* (Fr.) Dyko & B. Button, *Epicoccum nigrum* Link., *Sordaria lappae* Potebnia, *Curvularia* spp., *Dothiorella* spp, *Nothophoma quercina* (Sydow & P. Sydow) Q. Chen & L. Cai, *Phoma odoratissimi* Q. Chen, *Didymella aliena* (Fries) Q. Chen & L. Cai. It may be supposed that massive activation of pathogenic fungi is the result of weakening of plant immunity on the background of increasing abiotic stresses in Tbilisi over the years; which led to an imbalance between latent pathogens and host plants and eventually to the depressing consequences of trees dieback.

Keywords: conifers; endophytes; latent infection; pathogenic fungi; urban plantations

Literary data from recent decades have clearly shown that large-scale dieback of trees is a global ecological problem worldwide (Kim et al. 2013; Weed et al. 2013; Pavlov 2015; Bußkamp et al. 2020). Based on the scientific analysis of reasons for tree massive dieback, global warming and drought in terrestrial biomes are named as the main causes of the event. Namely, the combination of drought and unusually high temperatures, the so-called "global change-type drought", also known as "hot drought", causes vegetation change on the Earth and massive death of trees (Jentsch et al. 2007; Smith 2011; IPCC 2012; Müller et al. 2018).

The tree dieback may be caused by both direct impact on physiological processes and indirect reasons: by the activation of pests and pathogens. The high probability of exacerbation of epidemics under the influence of drought stress in forest ecosystems has been proven. Moreover, it has been established that global climate change may also alter the degree of virulence of various pathogens (Linnakoski et al. 2017).

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Based on molecular-genetic methods, it has been established that there are complex relationships between the pathogens (Brader et al. 2017). Therefore, the study of multiple plant infections (co-infection) and complex pathosystems (host-pathogen, pathogen-pathogen and host-multiple pathogen) is very important. Experimental results have shown that the symptoms, course, and outcomes of a disease caused by the same group of pathogens may be different in each particular individual, in dependence on interaction between the pathogens (Tollenaere et al. 2016; Abdullah et al. 2017).

The problem of massive disease and drying of trees is urgent in Georgia as well. In recent years, the massive dieback of woody plants, especially conifers, in the capital of Tbilisi and its surroundings has become noticeable (Abdaladze et al. 2019).

Thus, study of the microbiological status of tree plants in the most endangered areas of Tbilisi and its surroundings is needed.

The aim of the study was to conduct a mycological study of coniferous plantations in Tbilisi and its surroundings to identify the causes of tree dieback and decline, and to assess the existing risks of further progression of the disease. Large-scale studies of plant multipathogenic infections have not been conducted in Georgia yet; thus, this investigation is the first large-scale study to identify the causes of disease and its risk assessment in conifers in this area.

MATERIAL AND METHODS

In summer 2020, 42 localities were selected for the collection of biological material in Tbilisi and its surroundings, taking into account the massive disease and dieback of conifers in different parts of the city (Table 1). A total of 1 192 samples were taken from 247 conifers (mainly artificial plantings) which belonged to 15 species: *Abies pinsapo* Boiss., *Cedrus atlantica* (Endl.) Manetti ex Carrière, *Cedrus deodara* (Roxb. ex D.Don) G.Don, *Chamaecyparis lawsoniana* (A.Murray) Parl., *Cupressus sempervirens* L., *Juniperus communis* L., *Juniperus horizontalis* Moench, *Juniperus virginiana* L., *Picea pungens* Engelm., *Pinus nigra* J.F.Arnold, *Pinus pinea* L., *Pinus strobus* L., *Platycladus orientalis* (L.) Franco, *Thuja occidentalis* L., *Taxus baccata* L.

The biological material was taken from trees with various degrees of disease, observing maximal sterility. Only living parts of the plant (twigs, needles, in case of pine – previous year's cones) were cut. At least three samples were taken from each selected plant. Herbarium material was placed in polyethylene bags, and specimens for microbiological testing were placed in sterile containers, labelled with location and plant species. The collected samples were delivered to the microbiological laboratory.

To prevent the contamination by "accidental" microflora, the surface of the samples was disinfected: the plant material was washed under running water, placed on a filter paper for drying and cut (observing sterility) with a lancet into 1 × 1 cm pieces. The cut fragments (needles, bark, internodes, cone scales) were first placed in 70% ethanol for 10 s, then in 20% sodium hypochlorite for 1.5 min. The treated samples were washed with distilled water for 5 min (Vujanovic et al. 2000; Strobel 2018). Isolation of mycoflora from the biological material was carried out according to a standard scheme (fungal isolation protocol) (Lagamayo 2015), which involve obtaining of primary inoculums and subsequently pure cultures. Incubation of strains was carried out on potato-dextrose agar (PDA) and universal nutrient areas (composition of the universal agar medium ($g \cdot L^{-1}$): wort – 0.5 L, tap water – 0.5 L, agar-agar – 21 g; pH 5.5–5.8; sterilization mode at 121 °C for 15 min), in a thermostat at 25–28 °C for 10–14 days.

Pure cultures were stored in sloped tubes, in a sterilized, universal nutrient medium, in a refrigerator at 4 °C.

The study of cultural-morphological properties of pure cultures was carried out by macromorphological and microscopic research methods. The specific identification of 55 isolates of supposed pathogens by a molecular-genetic PCR method was performed in the Agriculture and Nutrition Laboratory of Guelph University, Canada. Taq DNA polymerase (Qiagen) and Qiagen PCR Buffer for minimal optimization were used for PCR amplification of ITS regions with the primer pair ITS4 and ITS5 (White et al. 1990). The ITS sequences were extracted with the open source software ITSx to separate the ITS1 and ITS2 subregions from the fungal ITS sequences (Bengtsson-Palme et al. 2013). The ITS1 and ITS2 sequences were used for BLASTN (Zhang et al. 2000) searches against GenBank/NCBI (Sayers et al. 2011) to provide taxonomic identification. The sequences with 98% similarity and query coverage of 97% were set to constrict the species level (Arnold, Lutzoni 2007). ABI 3730 DNA Analyzer and ABI PrismTM 7500 FAST Sequence Detection System were used for analysis.

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Table 1. The list of studied localities

No.	Experimental species	GPS coordinates (N, E)	Locality
1	*Cedrus deodara, Cupresus sempervirens, Juniperus communis, Pinus nigra, Platycladus orientalis*	41.71316, 44.71561	Tskneti, near-highway forest
2	*Cedrus deodara, Cupresus sempervirens, Pinus nigra, Platycladus orientalis*	41.68821, 44.80451	Sololaki slope, near the monument of Mother of Georgia
3	*Cedrus deodara, Cupresus sempervirens, Pinus nigra, Platycladus orientalis*	41.69544, 44.79404	Slope of mount Mtatsminda
4	*Cupresus sempervirens, Pinus nigra*	41.688222, 44.793444	Kojori Ln. Near the road
5	*Cedrus deodara, Cupresus sempervirens, Pinus nigra, Juniperus communis, Platycladus orientalis*	41.699793, 44.754646	Surrounding territory of Turtle lake
6	*Abies nordmaniana, Cedrus deodara, Cupresus sempervirens, Pinus nigra, Juniperus communis, Pinus strobes, Taxux baccata*	41.709490, 44.778044	Vake, Chavchavadze ave. 1,3. The territory of Javakhishvili State University
7	*Pinus nigra*	41.713415, 44.746557	Vake, near the stadium
8	*Taxux baccata*	41.712813, 44.748651	Vake, Chavchavadze ave. near the Ilia State Univessity
9	*Cedrus deodara, Cupresus sempervirens, Thuja occidentalis* cult.	41.732990, 44.772024	Saburtalo, Gotua st. 22, a square
10	*Abies concolor, A. sp., Calocedrus deccurens* cult. aureovariegata, *Cedrus atlantica* cult. pendula, *Chamaecyparis lawsoniana, Picea abies* cult. inversa, *Picea pungens, P.* sp., *Pinus strobus, Taxux baccata, Thuja occidentalis*	41.719054, 44.751552	Saburtalo, the territory of the old Hippodrome; he nursery
11	*Cedrus deodara, Cupresus sempervirens, Pinus nigra, Platycladus orientalis*	41.71736, 4474569	Saburtalo, crossroad of Tamarashvili and Amiredjibi streets
12	*Cedrus deodara, Cupresus sempervirens, Pinus nigra, Platycladus orientalis*	41. 72121, 44.74652	Tamarashvili st. 13; the territory of Hippodrome
13	*Cupresus sempervirens, Pinus nigra, P. pinea*	41.713442, 44.766230	Saburtalo, Amiredjibi st.; near tennis court
14	*Cedrus deodara, Cupresus sempervirens, Pinus nigra*	41.719755, 44.713762	Saburtalo, Politkovskaia st.
15	*Picea orientalis*	41.733639, 44.761789	Saburtalo, Vazisubani st. 5
16	*Cedrus deodara, Cupresus sempervirens, Pinus nigra, Platycladus orientalis*	41.725002, 44.754653	Saburtalo, Kazbegi ave.; Godziashvili sqaure
17	*Cedrus deodara*	41.727752, 44.757454	Saburtalo, Vazha-pshavela ave. 29; near the Republican hospital
18	*Cedrus atlantica* cult., *C. deodara, Picea pungens, Pinus nigra*	41.729699, 44.767598	Saburtalo, cross-road of Pekini ave. and Panjikidze st.
19	*Cupresus sempervirens*	41.730853, 44.731000	Saburtalo, Nutsubidze III microdist.

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Table 1 to be continued

No.	Experimental species	GPS coordinates	Locality
20	*Cedrus deodara, Cupresus sempervirens, Platycladus orientalis, Taxus baccata*	41.713481, 44.780332	Saburtalo, Kostava st. 64; Zoo territory
21	*Cedrus deodara, Cupresus sempervirens, Picea pungens, Pinus nigra*	41.712199, 44.781906	Saburtalo, the territory of the heroes memorial
22	*Cedrus deodara, Cupresus sempervirens, Juniperus horizontalis, J.* sp. cult., *Picea pungens, Pinus nigra, Taxus baccata, Thuja occidentalis*	41.713933, 44.779070	Saburtalo, Kostava st. 68; near the teritory of Public Broadcaster of Georgia
23	*Cedrus deodara, Cupresus sempervirens*	41.714541, 44.784183	Saburtalo, the slope near the Circus
24	*Abies* sp., *Cedrus deodara, Cupresus sempervirens, Juniperus communis, Picea pungens, Pinus nigra*	41.730311, 44.854948	Chugureti, on the territory of dendrologic park
25	*Cedrus deodara, Cupresus sempervirens, Juniperus communis, Pinus nigra, Platycladus orientalis*	41.735868, 44.852173	Chugureti, on the territory of new Zoo
26	*Cupresus sempervirens, Juniperus communis, Picea pungens, Pinus nigra*	41.765167, 44.807993	Temqa, unknown heroes st.
27	*Cedrus deodara, Cupresus sempervirens, Pinus nigra, Platycladus orientalis,*	41.741540, 44.778829	Didube, Tsereteli ave. 118; Expo Georgia
28	*Cedrus atlantica, C. deodara, Cupresus sempervirens pyram. C. sempervirens, Juniperus virginiana, Picea pungens, Pinus strobes, P. pinea, P. nigra, Platycladus orientalis, Thuja occidentalis*	41.747991, 44.780691	Didube, near Eristavi st.
29	*Cedrus deodara, Picea pungens, Pinus nigra*	41.731121, 44.782416	Didube, Agladze st.
30	*Cedrus deodara, Cupresus sempervirens, Juniperus horizontalis*	41.751177, 44.789459	Guramishvili ave. near the former cinema house "Sakartvelo"
31	*Cedrus deodara, Picea pungens, Pinus nigra*	41.760590, 44.788439	Sanzona, Guramishvili ave. 11, 15, 17, 19
32	*Pinus nigra, Platycladus orientalis*	41.797280, 44.798885	Avchala, surroundings of Sarajishvili ave. 12
33	*Abies pinsapo, Cedrus deodara, Cupresus sempervirens, Pinus nigra*	41.796495, 44.800648	Avchala, Sarajishvili ave. 21; wine factory "Bagrationi"
34	*Cupresus sempervirens, Pinus nigra, Platycladus orientalis*	41.823376, 44.784368	Avchala, crossroad of Andronikashvili and Bichvinta streets
35	*Cedrus atlantica, Cupresus sempervirens, Pinus nigra*	41.828237, 44.772225	Avchala, on the crossroad of Andronikashvili and Skhvitori streets
36	*Abies pinsapo, Cedrus deodara, Cupresus sempervirens, Picea pungens, Pinus nigra, Platycladus orientalis, Thuja occidentalis*	41.697484, 44.832836	Isani, territory of the Peters-Paul cemetery
37	*Cedrus deodara, Picea pungens, Pinus nigra, P. pinea, Taxus baccata*	41.767308, 44.771757	Digomi park, Lubliana st.
38	*Cedrus deodara, Cupresus sempervirens, Platycladus orientalis*	41.806215, 44.770062	Digomi, agmashenebeli alley; surroundings of the Agrarian University
39	*Cedrus deodara, Pinus nigra,*	41.779302, 44.768809	Digomi, Agmashenebeli alley 209, Mari market

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Table 1 to be continued

No.	Experimental species	GPS Coordinates	Locality
40	*Cupresus sempervirens*	41.748773, 44.793091	Ponichala, Gogoberidze st.
41	*Cedrus deodara, Taxus baccata*	41.701287, 44.795627	Rustaveli ave. 25, the Opera House square
42	*Cedrus deodara, Pinus nigra*	41.695256, 44.919594	Orkhevi, the square

Information on the nomenclature, taxonomy, synonymy, and distribution of fungal species in Georgia was obtained from CABI databases (http://www.speciesfungorum.org/).

RESULTS

A total of 1 169 isolates of microscopic fungi were isolated. Thirty-four microscopic fungi were identified to the species level, 17 to the genus level, and one isolate to the family level. The identified taxa belong to 18 families of three divisions: Ascomycota, represented by 15 families and 33 species, Basidiomycota – by 1 family and 2 species and Zygomycota – by 2 families and 2 species (Table 2). Nine dominant species of microscopic fungi were identified according to the prevalence and frequency on tested plants (Tables 2 and 3): *Alternaria alternata* (Fr.) Keissl, *Sphaeropsis sapinea* (Fr.) Dyko & B. Button, *Epicoccum nigrum* Link., *Sordaria lappae* Potebnia, *Curvularia* spp. (*C. spicifera* (Bainier) Boedijn, and *C. inaequalis* (Shear) Boedijn), *Dothiorella* spp. (*D. iberica* A.J.L. Phillips, J. Luque & A. Alves, and *D. gregaria* Succ.), *Nothophoma quercina* (Sydow & P. Sydow) Q. Chen & L. Cai, *Phoma odoratissimi* Q. Chen, *Didymella aliena* (Fries) Q. Chen & L. Cai. Eight of them are recognized pathogens of different severity (Agrios 2005).

Alternaria alternata. (Fr.) Keissl is the first-line dominant, which was isolated from 95% of the studied plants. Accordingly, the frequency of its occurrence was also highest among the total number of isolates (Tables 2 and 3).

The dangerous pathogen *Sphaeropsis sapinea* (Fr.) Dyko et Sutton was present in 39% of the total number of tested plants. It was observed in the mycoflora of all tested conifers, except *Chamaecyparis lawsoniana* and *Juniperus horizontalis.* It accounts for 11.2% of the total number of isolates (Tables 2 and 3).

Epicoccum nigrum Link. – 127 strains of this fungus were isolated from 55% of the tested plants (Table 2 and 3). According to PCR-diagnostics, with 99.8% similarity they belonged to *E. nigrum, E. layuense, E. tritici* and *E. poae*. However, they were closest to *E. nigrum* in terms of cultural-morphological characteristics.

Sordaria lappae Potebnia – 122 strains of this fungus were isolated from 42% of the studied conifers (Tables 2 and 3).

Curvularia spp. – 116 strains of the fungus were isolated from 44% of the studied plants (Tables 2 and 3), which belong to two closely related species: *C. spicifera* (Bainier) Boedijn, and *C. inaequalis* (Shear) Boedijn. Their pathogenic properties and degree of virulence are similar (Ayoubi et al. 2017). So, they are referred to in the text as *Curvularia* spp.

Two species of the genus *Dothiorella – D. iberica* A.J.L. Phillips, J. Luque & A. Alves and *D. gregaria* Succ. were detected in 21% of the plants tested (Table 3). Though *D. gregaria* strains were isolated only in a few cases, these two species of the genus *Dothiorella* in the text are referred to as *Dothiorella* ssp.

Representatives of the closely related species *Nothophoma quercina* (Sydow & P. Sydow) Q. Chen & L. Cai, *Phoma odoratissimi* Q. Chen and *Didymella aliena* (Fries) Q. Chen & L. Cai were found with the lowest frequency among the dominant pathogens – 5.3, 4.6 and 4.6% of the total number of isolates, respectively (Table 2). But if we consider that all three species cause one disease – phomosis, they can also be considered in the dominant category. Representatives of these genera were isolated from 57% of the total tested plants (Table 3).

As for non-dominant mycoflora, both pathogenic and beneficial species were found among them (Table 2). In total 212 strains of non-dominant fungi with sporadic distribution were isolated from the tested

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Table 2. Microscopic fungi in coniferous plants of Tbilisi and its surroundings and frequency of their occurrence (%)

	No.	Species of microscopic fungi (accepted name)	Family (division)	Number of isolates	FI (%)	Coniferous species
Dominant fungi	1	*Alternaria alternata* (Fr.) Keissl. group	Pleosporaceae (Ascomycota)	230	19.7	All tested plants
	2	*Sphaeropsis sapinea* (Fr.) Dyko & B. Button	Botryosphaeriaceae (Ascomycota)	131	11.2	All tested plants ex. *C. lawsoniana* and *J. horizontalis*
	3	*Epicoccum nigrum* Link	Didymellaceae (Ascomycota)	127	10.9	All tested plants
	4	*Sordaria lappae* Potebnia	Sordariaceae (Ascomycota)	122	10.4	All tested plants ex. *P. strobus, T. occidentalis*
	5	*Curvularia* spp. – *Curvularia spicifera* (Bainier) Boedijn, *Curvularia inaequalis* (Shear) Boedijn	Pleosporaceae (Ascomycota)	116	9.9	All tested plants ex. *J. communis, P. pinea*
	6	*Dothiorella* spp. – *Dothiorella iberica* A.J.L. Phillips, J. Luque & A. Alves, D. gregaria Sacc.	Botryosphaeriaceae (Ascomycota)	65	5.6	*C. deodara, C. sempervirens, J. communis, J. virginiana, P. pungens, P. nigra, P. orientalis, T. baccata, T. occidentalis*
	7	*Nothophoma quercina* (Syd. & P. Syd.) Qian Chen & L. Cai	Didymellaceae (Ascomycota)	62	5.3	*C. atlantica, C. deodara, C. sempervirens, J. communis, J. virginiana, P. pungens, P. nigra, P.orientalis, T. baccata, T. occidentalis*
	8	*Phoma odoratissimi* Qian Chen	Didymellaceae (Ascomycota)	54	4.6	*C. atlantica, C.deodara, C. sempervirens, P. nigra, P. strobus, P. orientalis, T. baccata, T. occidentalis*
	9	*Didymella aliena* (Fr.) Qian Chen & L. Cai	Didymellaceae (Ascomycota)	54	4.6	*C. atlantica, C. deodara, C. sempervirens, J. horyzontalis, P. nigra, P.strobus, P. orientalis, T. baccata, T. occidentalis*
Nondominant fungi	10	*Aspergillus niger* Tiegh.	Aspergillaceae (Ascomycota)	18	1.5	*A. pinsapo, C. atlantica, C. sempervirens, C. deodara, J. communis, P. nigra, P. orientalis, P. pungens, P. strobus*
	11	*Trichoderma citrinoviride* Bissett	Hypocreaceae (Ascomycota)	14	1.2	*C. sempervirens, C. deodara, P. orientalis, P. nigra, P. pungens*
	12	*Fusarium brachygibbosum* Padwick	Nectriaceae (Ascomycota)	11	0.9	*C. deodara, J. communis, P. nigra, P. pungens*
	13	*Alternaria infectoria* E.G. Simmons group	Pleosporaceae (Ascomycota)	11	0.9	*C. sempervirens, P. pungens, P. orientalis, P. nigra, T. occidentalis*
	14	*Cladosporium limoniforme* Bensch, Crous & U. Braun	Cladosporiaceae (Ascomycota)	10	0.9	*C. atlantica, C. deodara, P. nigra, P. orientalis, P. strobus, P. pinea, T. occidentalis*
	15	*Bipolaris cynodontis* (Marignoni) Shoemaker	Pleosporaceae (Ascomycota)	9	0.8	*C. deodara, C. sempervirens, P. nigra*
	16	*Mucor Racemosus* Fresen.	Mucoraceae (Zygomycota)	9	0.8	*C. deodara, C. sempervirens, P. nigra*

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Table 2 to be continued

	No.	Species of microscopic fungi (accepted name)	Family (division)	Number of isolates	FI (%)	Coniferous species
Nondominant fungi	17	*Fusarium equiseti* (Corda) Sacc.	Nectriaceae (Ascomycota)	9	0.8	*C. atlantica*, *C. deodara*, *C. sempervirens*, *P. nigra*, *P. pungens*
	18	*Botryosphaeria dothidea* (Moug.) Ces. & De Not	Botryosphaeriaceae (Ascomycota)	8	0.7	*C. atlantica*, *C. deodara*, *C. sempervirens*, *J. virginiana*, *P. nigra*, *P. orientalis*, *P. pinea*, *P. strobus*, *T. occidentalis*,
	19	*Preussia lignicola* (W. Phillips & Plowr.) Kruys	Sporormiaceae (Ascomycota)	7	0.6	*C. deodara*, *P. nigra*, *C. sempervirens*
	20	*Neurospora intermedia* F.L. Tai	Sordariaceae (Ascomycota)	7	0.6	*C. deodara*, *C. sempervirens*, *P. nigra*, *T. occidentalis*,
	21	*Chaetomium globosum* Kunze	Chaetomiaceae (Ascomycota)	6	0.5	*C. sempervirens*, *P. orientalis*, *P. nigra*
	22	*Xylaria cubensis* (Mont.) Fr.	Xylariaceae (Ascomycota)	6	0.5	*A. pinsapo*, *C. deodara*, *P. pungens*, *C. sempervirens*
	23	*Choanephora infundibulifera* (Curr.) D.D. Cunn.	Choanephoraceae (Zygomycota)	5	0.4	*C. atlantica*, *P. nigra*, *P. orientalis*
	24	*Sporormiella minima* (Auersw.) S.I. Ahmed & Cain	Sporormiaceae (Ascomycota)	5	0.4	*C. sempervirens*, *P. nigra*, *P. strobus*
	25	*Phanerochaete sordida* (P. Karst.) J. Erikss. & Ryvarden	Phanerochaetaceae (Basidiomycota)	4	0.3	*C. atlantica*, *C. sempervirens*, *P. orientalis*
	26	Epicoccum sp.	Didymellaceae (Ascomycota)	4	0.3	*C. atlantica*, *C. sempervirens*, *C. deodara*
	27	*Arthrinium sacchari* (Speg.) M.B. Ellis	Apiosporaceae (Ascomycota)	4	0.3	*C. deodara*, *P. orientalis*, *P. nigra*
	28	*Penicillium* sp.	Aspergilaceae (Ascomycota)	2	0.2	*C. deodara*, *P. nigra*
	29	*Alternaria* sp.	Pleosporaceae (Ascomycota)	2	0.2	*T. baccata*, *P. orientalis*
	30	*Sydowia polyspora* (Bref. & Tavel) E. Müll	Dothioraceae (Ascomycota)	2	0.2	*P. pinea*, *P. strobus*
	31	*Coniothyrium* sp.	Leptosphaeriaceae (Ascomycota)	2	0.2	*P. nigra*
	32	*Fusarium citricola* Guarnaccia, Sand.-Den. & Crous	Nectriaceae (Ascomycota)	2	0.2	*P. nigra*
	33	*Trichothecium roseum* (Pers.) Link	Incertae sedis	2	0.2	*P. nigra*
	34	*Nigrospora oryzae* (Berk &Broome) Petch	Apiosporaceae (Ascomycota)	1	0.1	*P. orientalis*
	35	*Hyphodermella rosae* (Bres.) Nakasone	Phanerochaetaceae (Basidiomycota)	1	0.1	*C. sempervirens*
	36	Nonidentified species		47	4.0	Different species
		Total		1 169	100	

FI – the frequency of occurrence of microscopic fungus in the total number of isolates – the ratio of the number of isolates of a particular strain to the total number of isolated cultures in %

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Table 3. The frequency of occurrence (FPS) of the dominant pathogenic fungi in conifers by species (%)

No.	Plant species	Microscopic fungus								
		Alternaria alternata group	*Sphaeropsis sapinea*	*Epicoccum nigrum*	*Sordaria lappae*	*Curvularia* sp.	*Dothiorella* spp.	*Nothophoma quercina*	*Phoma odoratissimi*	*Didymella aliena*
1	*Abies pinsapo*	100	25	25	50	75	0	0	0	0
2	*Cedrus atlantica*	89	67	67	56	11	0	56	11	22
3	*Cedrus deodara*	98	30	53	44	49	26	21	42	21
4	*Chamaecyparis lawsoniana*	100	0	25	75	100	0	0	0	0
5	*Cupressus sempervirens*	90	50	57	66	45	43	36	21	26
6	*Juniperus communis*	100	25	75	25	0	50	50	0	0
7	*Juniperus horizontalis*	100	0	100	50	50	0	0	0	50
8	*Juniperus virginiana*	100	50	50	50	50	50	50	0	0
9	*Picea pungens*	95	32	58	32	47	37	16	0	5
10	*Pinus nigra*	93	93	38	47	52	10	14	22	24
11	*Pinus pinea*	100	67	33	33	0	0	0	0	33
12	*Pinus strobus*	100	25	100	0	25	0	0	75	0
13	*Platycladus orientalis*	90	52	57	71	48	38	33	14	29
14	*Taxus baccata*	78	33	56	33	56	33	33	33	56
15	*Thuja occidentalis*	100	43	29	0	57	29	43	14	0
	Total	95	39	55	42	44	21	23	16	18

FPS – the ratio of the number of conifers infected with a given strain to the total number of conifers in percent

plants. Twenty-eight species were identified. Their share in the total number of isolates was 14%.

DISCUSSION

It is no longer disputed that the internal environment of plants, like that of animals, is not sterile and is inhabited by microscopic organisms (Mishra et al. 2014). An outwardly completely healthy and normally growing plant can host one or more species of microorganisms. They inhabit plant tissues and are integral components of their micro-ecosystem. It has been established that many of these endophytes are virulent pathogens, with limited pathogenic activity, and may latently exist in healthy plant leaves and stems for years (Carroll 1988; Schulz, Boyle 2005; Jia et al. 2016).

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A. alternata is a potent plant pathogen, with more than 4 000 host species; it causes great damage to agricultural products and it is a powerful allergen. *Alternaria* is a ubiquitous fungus that can be present in the host plant as both pathogenic and non-pathogenic agent, which is responsible for the production of specific host-dependent toxins (Grunden et al. 2001; Ribeiro et al. 2020). *S. sapinea* is a widespread pathogenic fungus (Africa, Asia, Europe, America). It is distinguished by high aggressiveness; it causes wilting of the apex and it can be latent in the host plant for years. The pathogen poses a great threat to both young and old plants. Unfavourable environmental conditions (drought, high temperature) contribute to the spread of the disease. Plants with insect-damaged bark and pines are easily infected with *diplodiosis*. The fungus infects many genera of conifers: *Abies*, *Cupressus*, *Larix*, *Pinus*, *Picea*, *Thuja*, *Juniperus* (Müller et al. 2018; Bußkamp et al. 2020). *E. nigrum* is a widespread (ubiquitous) non-systemic saprophyte fungus that under certain conditions becomes a facultative parasite of plants and causes the disease epicoccosis. In case of massive spread it brings severe consequences; the fungus is a risky factor for human and animal health. *E. nigrum* is considered to be one of the most important fungal allergens. It synthesizes the allergen Epip1. In sensitive people it causes allergic respiratory disorders, respiratory diseases, rhinitis, sinusitis, asthma (Noble et al. 1997). The pathogenic properties of *S. lappae* and degree of virulence have not been fully studied. According to the literature, the genus *Sordaria* in woody plants is considered as a weak pathogen (Ivanová et al. 2016). The genus *Curvularia* is widespread and includes saprophytes, as well as facultative parasites of plants, animals and humans. In plants it causes leaf rot and leaf burn (Krizsán et al. 2015; Kusai et al. 2016). One of the species revealed in our samples, *C. inaequalis*, produces strong-acting mycotoxins – pyrenocine A and B; it causes inhibition of plant growth and necrosis of leaves (Kim et al. 2000*).* Representatives of the genus *Dothiorella* (*D. gregaria* Sacc., *D. iberica* A.J.L. Phillips, J. Luque & A. Alves) are dangerous pathogens of woody plants and cause the wilting of needles, branches, twigs, ulcers on the stem, the wilting of the whole plant (Dissanayake et al. 2016). *D. iberica* is a relatively newly described species. It is an anamorph of *Botryosphaeria iberica*. From literary sources it is known as a serious plant pathogen of both wild and cultivated vines (*Vitis sylvestris*), hop-hornbeam (*Ostrya*) and others (Phillips et al. 2005). It is especially dangerous when the teleomorphic form of *Dothiorella — Botryosphaeria ribis* develops on the same plant (Mehl et al. 2013). However, the presence of this agent was not observed in our study. Three closely related species *Nothophoma quercina* (Sydow & P. Sydow) Q. Chen & L. Cai, *Phoma odoratissimi* Q. Chen and *Didymella aliena* (Fries) Q. Chen & L. Cai belong to the family Didymellaceae, which includes anamorphs and teleomorphs described as different species, saprophytes, weak and strong parasites, as well as superparasites (Sutton 1980; Deb et al. 2020).

Particular attention should be paid to the detection of such pathogens as *Botryosphaeria dothidea*, *Fusarium brachygibbosum*, *F. equiseti*, *Preussia lignicola*, *Bipolaris cynodontis*. Also noteworthy is the classic endophyte *Sydowia polyspora*. According to some researchers, it is found in the dominant status only in the endophytic microflora of healthy pines (Bußkamp et al. 2020). Thus, the low frequency of *S. polyspora* in Tbilisi and its surrounding plantations should be the indication of the unhealthy condition of the capital's pines.

The particular scarcity of basidiomycetes in the microflora of the tested plants should be explained by the fact that the method used in the experiment (especially surface sterilization of the branches) and the nutrient area were mainly focused on the cultivation of microscopic fungi. In addition, it is known that microscopic fungi are characterized by much faster growth ability; that is why they occupy a niche faster than Basidiomycota (Zheng et al. 2020).

Due to the very low frequency of individual non-dominant species, they could not play a significant role in the large-scale dieback of trees; therefore, the focus was on the dominant species to determine the causes of the problem.

While comparing the microflora of the studied species no significant differences were found. In almost all tested species, all eight dominant pathogens were present together, with a few exceptions (Tables 2 and 3). Thus, co-infection – coexistence of multiple pathogens occurred in all studied conifers.

Establishment of possible relationships between plant pathogens is the object of intensive research in scientific circles today (Abdullah et al. 2017). However, the main purpose of our experiment was to determine the causes of conifer dieback through-

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out the city and not to elucidate the relationships between pathogens. Though, the obtained results gave us at least some idea from this point of view. The first-order dominant among pathogens *A. alternata* is found in almost all species of conifers with almost equal frequency (78–100%), which is an indicator of high competitiveness of this species compared to other identified fungi. Presumably it "owns" a defined niche quickly, however, it does not rule out the coexistence of other species. In the latent phase of existence in the host plant, *Alternaria* produces substances that cause structural modification of the mycelium of *Plasmopara viticola* inhabiting the same plant, and inhibits the sporulation of the pathogen Oomycete; thus even rendering a useful service to the host plant (Ribeiro et al. 2020). However, under unfavourable physiological conditions *A. alternata* becomes an aggressive pathogen in the host plant (Ribeiro et al. 2020). One of the mechanisms by which the biotrophic fungus is transformed into a necrotroph lies in the synthesis of non-ribosomal toxic peptides. With these toxins it kills host cells and uses them for food (Ribeiro et al. 2020).

S. sapinea is a pathogen of conifers and especially of pines in many countries of the world, in temperate and subtropical zones. It is latently present in various pine organs for a long period (Bihon et al. 2011). As far as various stress factors (drought, hail, pests, etc.) more or less weaken the plant, the latent pathogen turns into an aggressive patho-form and begins an active development; which is ultimately manifested by symptoms of the disease (Müller et al. 2018)

The frequency of another dangerous pathogen *Dothiorella* was highest in *Cupressus sempervirens*, *Juniperus communis*, *J. virginiana*, *Platicladus orientalis* and *Picea pungens* (Table 3). It should be noted that *Sphaeropsis* and *Dothiorella* are so dangerous pathogens of conifers (and not only of them) that they can cause the plant death (Smahi et al. 2017). The coexistence of these two severe pathogens was detected in most tested conifers. Presumably, they "play" a leading role in the massive drying of coniferous plants in the city against the background of the coexistence of other pathogens.

From the table it is clear that *Chamaecyparis lawsoniana* is a particularly favourable environment for *Curvularia* (Table 3). It was isolated from all the tested trees of this conifer. The genus *Curvularia* is known for its ability to produce mycotoxins (Rout et al. 1989), which may inhibit the genus *S. sapinea*, *D. iberica*, and *Phoma* in this species.

The increased frequency of pathogens in conifers indicates that from a latent state they went to an "aggressive" one. Based on the analysis of the obtained results, it may be assumed that the activation of pathogenic fungi from the latent state had certain reasons. The main purpose of the experiment was to determine these reasons.

Many factors need to be considered here, including the important interactions between the host plant and its microbiocoenosis.

It is generally known that the mutualistic relationship is often formed between endophytes and the host plant. Microorganisms that settle on the vegetative parts of the host remain metabolically inactive for a long time; but when host plant tissues are damaged by an animal or insect, or even exposed to some abiotic stress, the growth of "inducible mutualists" is activated, and they even begin to produce toxins against harmful factors (Carroll 1988). Thus, the seemingly "benevolent" relationship of the latent pathogen and its host plant is not so simple and at some point of the coexistence it may change from mutualistic to cryptic commensalism, or even to latent and virulent pathogenicity. Researchers believe that such a change (depending on environmental conditions, host inheritance, microorganism species, and form of interaction) is a common phenomenon in balancing the symbiotic relationships (Stergiopoulos, Gordon 2014).

It has been established by a number of authors that a balanced state between the virulence of latent pathogens and the host plant protection reactions prevents disease development in the plant. As far as the balance established between antagonists shifts to the fungal side (which, for example, can be caused by the host plant stress), the pathogen is activated and eventually it leads to complete destruction of the host (Schulz, Boyle 2005; Newton et al. 2010). Based on the above speculations, abiotic stress is the main factor in the activation of latent pathogens; in case of Tbilisi — the dire ecological situation in the capital.

It is known that Tbilisi is included in the list of high-impact municipalities, where in the near future (for 2021–2050) a sharp deterioration of living conditions is expected due to changes in the absolute maximum temperature and the annual average number of hot days (The Georgian Road Map on Climate Change Adaptation 2016). In terms of studying the climate change

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it was revealed that the average annual temperature in Tbilisi has increased by 0.4 °C (compared to the 60s of the last century). An increase in the intensity of heat waves, and the tendency of intensification of the effect of so-called thermal islands have also been noted, which in addition to the chaotic urban development of Tbilisi, further aggravates the already stressful environmental conditions for plants (Georgia's Third National Communication to the UN Framework Convention on Climate Change 2015). There is a very serious situation and pessimistic forecast in terms of average annual precipitation as well. Tbilisi is a low-rainfall city with average annual precipitation of 560 mm. In the last decade, in Kvemo Kartli, which borders Tbilisi to the east, south and partly to the west, there has been a 5% decrease in average annual rainfall, which is an indicator of deteriorating living conditions for plants (Elizbarashvili et al. 2017).

Along with high temperatures and water shortages, the severe ecological condition of the city is further aggravated by air pollution with harmful gases and dust particles (Annual of Air Pollution in the Territory of Georgia 2017).

CONCLUSION

The mycological study of coniferous plantations in Tbilisi and its surroundings revealed the great diversity of endomycoflora. Eight dominant species were recognized as pathogens of different severity. As for non-dominant mycoflora, both pathogenic and beneficial species were found among them. Particular attention should be paid to the detection of such pathogens as *Botryosphaeria dothidea*, *Fusarium brachygibbosum*, *Fusarium equiseti*, *Preussia lignicola*, *Bipolaris cynodontis*. Due to the very low frequency of individual non-dominant species, they could not play a significant role in the large-scale dieback of conifers. It may be supposed that weakening of the immunity of conifers on the background of increasing abiotic stresses in Tbilisi over the years has led to an imbalance between latent pathogens and host plants and a massive activation of pathogenic fungi; this eventually led to the depressing consequences of tree dieback.

The phenomenon of massive disease and wilting of coniferous species in the capital is a warning for the near future: if some measures are not taken to mitigate the situation, we will get the same result with deciduous trees.

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