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Phytopathogenic fungi in forest nurseries of Middle Siberia

Marina A Sheller [1-2],
Elena A Shilkina [1],
Aleksey A Ibe [1],
Tatyana Y Razdorozhnaya [1],
Tatyana V Sukhikh [1]

The paper presents the results of phytopathogenic fungi determination in bare-root forest nurseries of Middle Siberia. Genetic analysis of pathogenic microflora of *Pinus sylvestris* L., *Pinus sibirica* Du Tour and *Picea obovata* Ledeb. seedlings allowed identification of 17 genera of micromycetes: *Phoma* Sacc., *Lophodermium* Chevall., *Sclerophoma* Höhn. (teleomorph *Sydowia* Bres.), *Cladosporium* Link, *Alternaria* Nees, *Typhula* (Pers.) Fr. etc. Most frequently detected fungi represented genera *Phoma* (23.7 %) and *Lophodermium* (23.6 %). *Pinus sylvestris* L. seedlings harboured the highest diversity of fungal taxa. Seven genera of microscopic fungi (*Phoma* sp., *Didymella* sp., *Alternaria* sp., *Cladosporium* sp., *Lophodermium* sp., *Gremmenia* sp., *Sclerophoma* sp.) were detected in all studied forest zones: taiga, forest-steppe and Southern-Siberian mountain. The obtained results demonstrate the usefulness of DNA analysis for the identification of phytopathogenic fungi in forest nurseries of Middle Siberia with several implications for increasing the efficacy of forest management.

Keywords: Forestry, Forest Nurseries, Phytopathogens, Conifers, DNA analysis, ITS Region, Phytopathological Monitoring

Introduction

The quality of the reproductive material for afforestation/reforestation activities is one of the most important problems of the Russian forest sector at the present time (Bespalova et al. 2019). Almost 200 forest nurseries in Middle Siberia provide planting stock for the state afforestation program. Most seedlings are grown using bare-root cultivation system. As a rule, the production of planting material in such a way is associated with some losses which can be caused by a number of abiotic and biotic factors (Ndobe 2012, Keča 2016). The abiotic disorders could be linked to inappropriate cultivation practices or environmental factors, and among a wide variety of biotic factors, pathogenic fungi are important stressors affecting tree health (Desprez-Loustau et al. 2006, Lilja et al. 2010).

Significant losses of coniferous seedlings due to the spread of fungal infections are observed every year in forest nurseries of Middle Siberia. A violation of homeostasis of microbial cenoses in forest nursery soils, associated with an adverse environmental situation is one of the causes of fungal epiphytotics in the region (Shilkina 2004). The study of the microbial community and the combination of a wide range of environmental factors that determine its stability and variability deserves attention concerning possible prediction of the spread of certain groups of microorganisms, in particular plant pathogenic fungi.

It is rather difficult to define the border between fungal infections because they usually begin with similar symptoms and their visual inspection is often unreliable (Stenström et al. 2014, Shestibratov et al. 2018). In this case the molecular genetic analysis is one of the most effective methods of early diagnostics and taxonomical determination of pathogens in forest nurseries. It allows detecting pathogen DNA with high accuracy and at an early stage of the disease development (Kernaghan et al. 2003, Menkis 2005, Baranov et al. 2010, Okorski et al. 2019). The goal of our study was to identify phytopathogenic fungi on *Pinus sylvestris* L., *Pinus sibirica* Du Tour and *Picea obovata* Ledeb. seedlings in 29 bare-root forest nurseries of Middle Siberia by sequencing the internal transcribed spacer of the fungal ribosomal DNA (ITS rDNA).

Material and methods

The microflora of 1410 Scots pine (*Pinus sylvestris* L.), 600 Siberian stone pine (*Pinus sibirica* Du Tour) and 600 Siberian spruce (*Picea obovata* Ledeb.) diseased seedlings from 29 forest nurseries of Middle Siberia was studied over a period of 6 years. The forest nurseries are located in three forest zones: taiga, forest-steppe, Southern-Siberian mountain (Consortium Kodeks 2020 - Tab. 1, Fig. 1). Before sampling, the forest nurseries were examined and then 30 seedlings with disease symptoms (yellowing, browning, damping off, etc.) were drawn from the entire area in each age group (Innovations in Russia 2013). The plants without signs of damage were used as control samples. Additionally, 435 control samples were analyzed.

The laboratory analyses generally followed the study by Menkis et al. (2006). In brief, DNA from the damaged plant tissues (needles) was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle & Doyle 1990). The universal primers ITS1 and ITS4 were used to amplify the ITS1-5.8S/ITS2 regions between the 18S and 28S nuclear rDNA (White et al. 1990). PCR was performed using GenPak® PCR Core Kit (Laboratory Isogen Ltd., Russia); 20 µl of the reaction mix were prepared including 2.5 µl of each primer (20 µM) and 5 µl of DNA template. The amplifi-

(1) Department of Monitoring of Forest Genetic Recourses, Branch of the Russian Centre of Forest Health - Centre of Forest Health of Krasnoyarsk krai, Akademgorodok 50a/2, 660036 Krasnoyarsk (Russia); (2) Reshetnev Siberian State University of Science and Technology, Krasnoyarskii rabochii prospect 31, 660037 Krasnoyarsk (Russia)

@ Marina A Sheller (maralexsheller@mail.ru)

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Tab. 1 - Twenty-nine forest nurseries in Middle Siberia where diseased seedlings of *Pinus sylvestris* L., *Pinus sibirica* Du Tour and *Picea obovata* Ledeb. were investigated using direct sequencing method. Forest zones: (T) Taiga; (Fs) Forest-steppe; (SSm) Southern-Siberian mountain.

No	Forest nursery	Forest zone	Fungal taxa
1	Abanskiy	T	*Lophodermium seditiosum, Phoma* sp., *Gremmenia* sp., *Typhula intermedia, Sclerophoma* sp.
2	Bolsheuluyskiy	T	*Alternaria tenuissima, Alternaria alternata, Alternaria* sp., *Phoma* sp., *Herpotrichia* sp.
3	Dolgomostovskiy	T	*Cladosporium* sp., *Phoma* sp., *Didymella macrostoma*
4	Kemskiy	T	*Typhula intermedia, Lophodermium seditiosum, Alternaria* sp.
5	Maklakovskiy	T	*Gremmenia infestans, Phoma* sp., *Lophodermium seditiosum*
6	Pirovskiy	T	*Alternaria* sp.
7	Reshotinskiy	T	*Cladosporium* sp., *Lophodermium seditiosum, Gremmenia infestans, Phoma* sp., *Rhizoctonia* sp., *Typhula* sp.
8	Taseevskiy	T	*Cladosporium* sp., *Lophodermium seditiosum, Lophodermium* sp., *Phoma* sp., *Rhizoctonia solani, Sclerophoma* sp.
9	Tinskiy	T	*Phoma* sp., *Typhula* sp.
10	Tyukhtetskiy	T	*Herpotrichia juniperi*
11	Bogradskiy	Fs	*Phoma* sp.
12	Dzerzhinskiy	Fs	*Sclerophoma* sp.
13	Ilanskiy	Fs	*Lophodermium seditiosum, Sclerotinia* sp.
14	Kemchugskiy	Fs	*Botrytis* sp., *Cladosporium* sp., *Coleosporium* sp., *Didymella macrostoma, Lophodermium seditiosum, Lophodermium* sp., *Phoma* sp.
15	Maganskiy	Fs	*Lophodermium seditiosum, Typhula* sp.
16	Mininskiy	Fs	*Didymella pomorum, Phoma* sp.
17	Sukhobuzimskiy	Fs	*Alternaria* sp., *Phoma* sp.
18	Tayezhnyy	Fs	*Lophodermium seditiosum, Phoma* sp., *Typhula* sp.
19	Talovskiy	Fs	*Alternaria* sp., *Cladosporium* sp., *Lophodermium seditiosum, Gremmenia* sp., *Phoma* sp., *Gremmeniella abietina, Sclerophoma* sp., *Septorioides pini-thunbergii*
20	Trudnovskiy	Fs	*Botrytis* sp., *Cladosporium* sp., *Herpotrichia* sp., *Phoma* sp., *Sclerophoma* sp.
21	Tuimskiy	Fs	*Phoma* sp.
22	Uzhurskiy	Fs	*Didymella glomerata, Lophodermium seditiosum, Lophodermium* sp., *Phoma* sp.
23	Uyarskiy	Fs	*Phoma* sp.
24	Verkhnemanskiy	SSm	*Lophodermium seditiosum, Lophodermium* sp.
25	Goryachegorskiy	SSm	*Alternaria* sp., *Cladosporium* sp., *Didymella macrostoma, Epicoccum* sp., *Phoma* sp., *Rhizoctonia solani*
26	Ermakovskiy	SSm	*Alternaria alternata, Alternaria tenuissima, Cladosporium* sp., *Lophodermium pinastri, Lophodermium seditiosum, Gremmenia* sp., *Phoma* sp., *Sclerophoma* sp.
27	Kazyrskiy	SSm	*Phoma* sp.
28	Verkhnetashtypskiy	SSm	*Phoma* sp., *Rhizoctonia solani*
29	Shalinskiy	SSm	*Cladosporium herbarum, Cladosporium* sp., *Lophodermium seditiosum, Phoma* sp., *Ramularia* sp., *Sclerotinia nivalis*

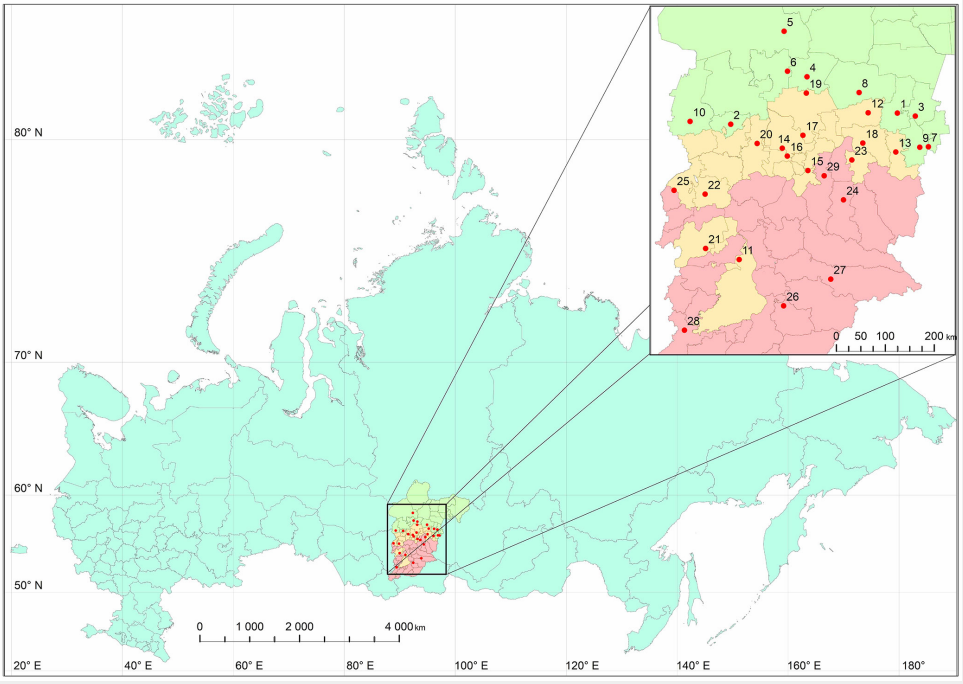

Fig. 1 - Map of Russia showing the locations of the investigated forest nurseries (numbered 1-29 as in Tab. 1) from which seedlings were collected. Forest nurseries are indicated by red dots. Taiga, forest-steppe and Southern-Siberian mountain zones are indicated by green, orange and pink, respectively.

cation cycle consisted of an initial denaturation step at 94 °C for 1 min, followed by 35 cycles of 94 °C for 15 sec, 60 °C for 20 sec, and 72 °C for 26 sec, and a final extension at 72 °C for 10 min. The PCR products were electrophoresed in a 2% agarose gel in Tris-borate-EDTA (TBE) (1×) buffer, stained with ethidium bromide and visualized under UV light. The PCR products were then excised and purified using the BioSilica Kit (BioSilica Ltd., Russia) following the manufacturer's instructions. Sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The DNA sequence analysis was performed on an ABI PRISM 310® genetic analyzer (Applied Biosystems, Foster City, CA, USA). For taxonomic identification sequences were submitted to the NCBI Genbank database (NCBI 2020). The criteria used for identification were: sequence coverage >80%, identity to species level 98-100%, identity to genus level 94-97%. Sequences not matching those criteria or lacking taxonomic names in the reference sequences were considered unidentified and therefore were not taken into analysis. The taxonomy of identified fungi was updated in accordance with the international database Index Fungorum (2020).

Results and discussion

Amplification of fungal ITS rDNA from 2610 diseased seedlings was successful for 2341 (89.7%) samples. Each of the PCR reactions produced one to three amplicons. Direct sequencing of all amplicons resulted in 2710 sequences. Among them, 1596 (58.9%) sequences were identified and taken into analysis. DNA analysis of 435 control samples resulted in 159 sequences. However, 72 (45.3%) of them were of low quality and were discarded from the analysis. Eighty-seven control samples contained genetic material of saprotrophic (*Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp.) and conditionally pathogenic fungi (*Phoma* sp., *Sclerophoma* sp., *Cladosporium* sp.).

The representatives of 17 genera of pathogenic and conditionally pathogenic fungi were identified in forest nurseries of Middle Siberia: *Phoma* Sacc. and *Didymella* Sacc., *Lophodermium* Chevall., *Sclerophoma* Höhn. (teleomorph *Sydowia* Bres.), *Cladosporium* Link, *Alternaria* Nees, *Typhula* (Pers.) Fr., *Herpotrichia* Fuckel., *Gremmenia* Korf., *Rhizoctonia* DC., *Sclerotinia* Fuckel, *Botrytis* P. Micheli ex Pers., *Coleosporium* Lév, *Epicoccum* Link, *Gremmeniella* M. Morelet (*Scleroderris* [Fr.] Bonord.), *Septorioides* Quaedvl., Verkley & Crous, *Ramularia* Sacc. Interestingly, 7 genera of these micromycetes were found in all studied forest zones, but most of them were present in nurseries located in forest-steppe zones (Tab. 2). Apart from this, several genera of mold fungi were also determined in infected seedlings: *Aspergillus* P. Micheli, *Penicillium* Link and *Rhizopus* Ehrenb. Such molds are likely part of the seedlings natural microflora or are contaminants from soil that accumulate on seedling shoots while lifting (Sutherland et al. 1991). In each of the nurseries pathogenic microflora was represented by 1-8 genera of microscopic fungi (Tab. 1). Ascomycetes were the most common fungi identified in the forest nurseries of Middle Siberia.

Scots pine seedlings harboured the highest diversity of fungal taxa (Fig. 2). Mostly these are the fungi of genera *Lophodermium* and *Phoma*, as well as *Typhula, Cladosporium, Alternaria, Sclerophoma, Rhizoctonia, Gremmenia, Didymella, Sclerotinia, Botrytis, Coleosporium, Gremmeniella.* Eleven genera of micromycetes were found on diseased seedlings of Siberian stone pine. Among them the most dominant are *Sclerophoma, Cladosporium, Herpotrichia* and *Phoma*. The studied microflora of Siberian spruce seedlings exhibited the lowest diversity of fungal taxa, with a predominance of *Phoma* sp., *Sclerophoma* sp. and *Alternaria* sp. Consequently, six genera of micromycetes were detected on diseased seedlings of all studied host tree species: *Alternaria, Cladosporium, Didymella, Gremmenia, Phoma* and *Sclerophoma.*

The analysis showed that fungi belonging to the genus *Phoma* (23.7%) were the most common phytopathogens found on diseased seedlings in forest nurseries of Middle Siberia (Fig. 3). They were detected on Scots pine, Siberian stone pine and Siberian spruce seedlings in 22 nurseries. *Phoma* species are ubiquitous soil inhabitants. The fungi invade seedlings from soil, usually through the lower needles, then infection spreads up the seedling crown, affecting needles until the seedling is defoliated (James 2012). *Phoma* spp. occur through-

Tab. 2 - Phytopathogens observed on *Pinus sylvesrtis* L., *Pinus sibirica* Du Tour and *Picea obovata* Ledeb. seedlings in different forest zones of Middle Siberia.

Fungal taxa	Forest zone			Host tree species		
	Taiga	Forest-steppe	Southern-Siberian mountain	*Pinus sylvesrtis*	*Pinus sibirica*	*Picea obovata*
Alternaria sp. (*A. alternata* [Fr.] Keissl.)	+	-	+	+	-	+
Alternaria sp. (*A. tenuissima* [Kunze] Wiltshire)	+	-	+	-	-	-
Alternaria sp.	-	+	+	-	+	-
Botrytis sp.	-	+	-	+	-	-
Cladosporium sp.	+	+	+	+	+	-
Cladosporium sp. (*C. herbarum* [Pers.] Link)	-	-	+	-	-	+
Didymella (*D. glomerata* [Corda] Q. Chen & L. Cai)	-	+	-	+	-	-
Didymella (*D. macrostoma* [Mont.] Q. Chen & L. Cai)	+	+	+	+	-	+
Didymella (*D. pomorum* [Thüm.] Q. Chen & L. Cai)	-	+	-	-	+	-
Epicoccum sp.	-	-	+	-	-	+
Gremmenia sp.	-	+	+	-	+	+
Gremmenia sp. (*G. infestans* [P. Karst.] Crous = *Phacidium infestans* P. Karst.)	+	-	-	+	-	-
Gremmeniella sp. (*G. abietina* [Lagerb.] M. Morelet = *Scleroderris lagerbergii* Gremmen)	-	+	-	+	-	-
Herpotrichia sp.	+	+	-	-	+	-
Herpotrichia juniperi (Sacc.) Petr.	+	-	-	-	-	+
Lophodermium sp. (*L. seditiosum* Minter, Staley & Millar)	+	+	+	+	+	-
Lophodermium sp. (*L. pinastri* [Schrad.] Chevall.)	-	-	+	-	+	-
Phoma sp.	+	+	+	+	+	+
Sclerophoma sp. (teleomorph - Sydowia Bres.)	+	+	+	+	+	+
Septorioides sp. (*S. pini-thunbergii* [S. Kaneko] Quaedvl., Verkley & Crous)	-	+	-	-	+	-
Typhula sp.	-	+	-	+	-	-
Typhula sp. (*T. intermedia* Appel & Laubert)	+	-	-	-	-	-
Sclerotinia sp.	-	+	-	+	-	-
Sclerotinia sp. (*S. nivalis* I. Saito)	-	-	+	-	-	+
Ramularia sp.	-	-	+	-	+	-
Rhizoctonia sp.	+	-	-	+	-	-
Rhizoctonia sp. (*R. solani* J.G. Kühn)	-	-	+	-	+	-
Coleosporium sp.	-	+	-	-	-	-

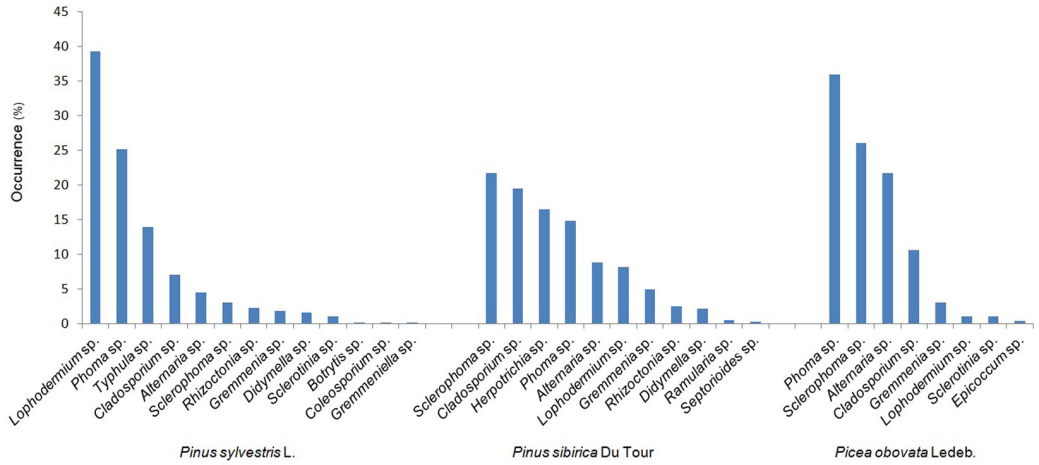

Fig. 2 - The occurrence of the main phytopathogens on the seedlings of *Pinus sylvestris* L., *Picea obovata* Ledeb., *Pinus sibirica* Du Tour.

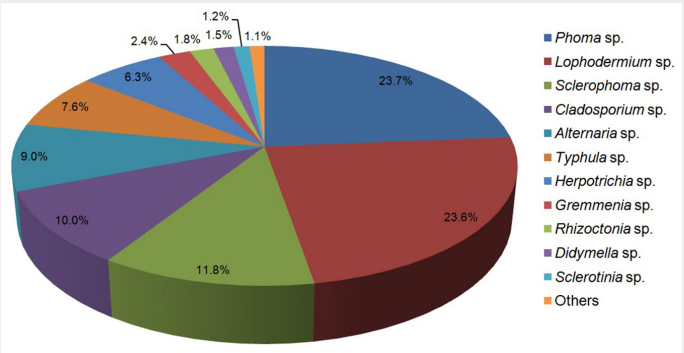

Fig. 3 - The occurrence of the main phytopathogens in 29 investigated forest nurseries in Middle Siberia.

out the world and can be of significant economic importance (Aveskamp et al. 2008, Seredich 2016). In forest nurseries of Middle Siberia *Phoma* species have not been previously diagnosed and therefore scarce information is available on the pathogenic fungi of this genus in the region.

The increase of the environmental extremes due to climate change may have caused *Phoma* fungi to become active in forest nurseries of Middle Siberia. As in many cases of poor health, environmental factors play a key role in the probability of infection and transmission of the pathogen (Lilja et al. 2010). Intensive cultivation practices also could lead to natural accumulation of the infection in forest nurseries. It should be noted that the identification of *Phoma* fungi in forest nurseries of Middle Siberia has become possible mainly due to the implementation of molecular genetic diagnostics into phytopathological monitoring of forest nurseries. Hence, the study of *Phoma* species biology, ecology and pathogenic activities in the microflora of coniferous seedlings is of great scientific and practical interest. Besides, a number of identified species of the genus *Didymella* (1.5%), such as *D. macrostoma, D. glomerata, D. pomorum*, were previously classified to the genus *Phoma*. It also indicates the need for accurate study of the taxonomy of *Phoma* species (Shilkina et al. 2018).

The second most frequently occurred phytopathogens found in 14 forest nurseries were *Lophodermium* fungi (23.6%). *Lophodermium* is a well-known fungal genus which occurs in pine needles (Ortiz-García et al. 2003, Millberg 2015). In the present study two species of this genus were identified in needles of Scots pine and Siberian stone pine seedlings, namely *L. seditiosum* and *L. pinastri*. Of these two species, only *L. seditiosum* is considered to be pathogenic, causing *Lophodermium* needle cast disease (Stenström & Ihrmark 2005, Lilja et al. 2010). *L. pinastri* is reported to be non-pathogenic fungus that lives endophytically within pine needles (Minter & Millar 1980). The obtained results are in agreement with another study (Grodnitskaya & Kuznetsova 2012), which showed that *L. seditiosum* and *L. pinastri* were widely spread on pine seedlings in forest nurseries of Middle and South Siberia.

Cladosporium (*C. Herbarum* – 10 %) and *Alternaria* (*A. alternata, A. Tenuissima* – 9 %) were also among the most common pathogens found in 9 and 7 nurseries, respectively. They were detected on all of the

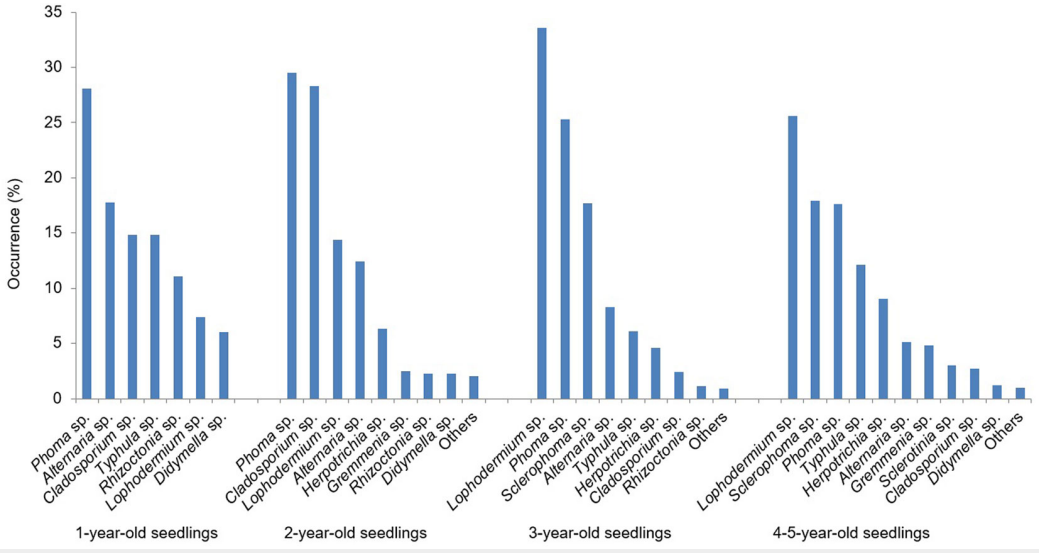

Fig. 4 - The occurrence of the main phytopathogens on coniferous seedlings of different age groups.

studied host tree species and in all forest zones. In addition, *Cladosporium* fungi were present with low abundance in the epiphytic microflora of healthy plants. *Alternaria* and *Cladosporium* are facultative parasites mainly associated with plants weakened by unfavorable climatic conditions (Yarmolovich et al. 2016). In forest nurseries they usually cause post-emergence damping-off (Procházková & Vlastislav 1991). It is worth to point out that in Middle Siberia the most common casual agents of post-emergence damping-off are *Fusarium* fungi (Yakimenko & Grodnitskaya 2000, Shilkina 2004). In the present study they might not have been detected by DNA analysis due to the poor quality of several obtained sequences.

Sclerophoma (11.8%), *Herpotrichia* (6.3%) and *Gremmenia* (2.4%) were detected in 6, 3 and 5 forest nurseries, respectively. Most of the nurseries were located in taiga and forest-steppe zones. Fungi of the genera *Sclerophoma* and *Gremmenia* were found on Scots pine, Siberian stone pine and Siberian spruce seedlings, while *Herpotrichia* sp. was detected only on Siberian stone pine and Siberian spruce seedlings. *Sclerophoma* sp. (teleomorph, *Sydowia* Bres.) was frequently determined on damaged plants and ranked third on the occurrence. In some cases the stems of seedlings infected by *Sclerophoma* had S-shaped bending which is one of the symptoms of the disease. *Sclerophoma* is reported to be a weak pathogen which occasionally causes serious damage of trees under stress (Kraj et al. 2009). Snow blight of conifers seedlings in surveyed nurseries was caused by *Gremmenia infestans* and *Herpotrichia juniperi*. In forest nurseries of Siberia it is a generally known disease which is successfully diagnosed by macro- and microscopic methods and there are specific recommendations for its prevention and control (Shilkina et al. 2018).

The typical genera of snow mold, *Typhula* (7.6%) and *Sclerotinia* (1.2%), were found on Scots pine and Siberian spruce seedlings in 6 and 2 nurseries, respectively. Most species of *Typhula* and *Sclerotinia* are low-temperature tolerant and are widely distributed in cold regions in the Northern Hemisphere (Hoshino et al. 2009, Matsumoto 2009). In Middle Siberia snow mold caused by *Typhula* sp., unlike *Sclerotinia* sp., is poorly studied on conifers and its identification has become easier only with the use of DNA analysis (Shilkina et al. 2018). Current research showed that the disease is widespread in forest nurseries located in taiga and forest-steppe zones. Consequently, the study of its agents is relevant for the development of reasonable methods of prevention and protection.

Among the rarely diagnosed phytopathogens of seedlings were *Rhizoctonia* fungi (1.8%). *Rhizoctonia solani* was identified on Scots pine and Siberian stone pine seedlings in 4 forest nurseries located in taiga and Southern-Siberian mountain zones. Rhizoctonia blight, caused by species of *Rhizoctonia*, occurs on many pine species and can lead to severe damages of seedlings in bare-root nurseries, *e.g.*, damping-off and rot of roots, stems, needles, and terminal buds (Starkey & Enebak 2012). In forest nurseries of Middle Siberia the fungi of the genus *Rhizoctonia* were only detected in soil, but their harmfulness for seedlings was not observed (Gromovykh et al. 2005). Single cases of occurrence were detected for *Botrytis* sp., *Gremmeniella* sp., *Coleosporium* sp. (Scots pine), *Septorioides* sp. (Siberian stone pine), *Epicccocum* sp. (Siberian spruce). Given the low frequency of occurrence these pathogens do not pose a strong threat and therefore do not have current economic significance in forest nurseries of Middle Siberia. The analysis of the presence of the main phytopathogens on seedlings of different age groups has revealed that the most predominant pathogens of one- and two-year-old plants are *Phoma* sp., *Alternaria* sp., *Cladosporium* sp. and *Typhula* sp.; three- four- and five-year-old seedlings were mostly affected by *Lophodermium* sp., *Phoma* sp., *Sclerophoma* sp. and *Typhula* sp. (Fig. 4).

In this study, the spectrum of phytopathogenic fungi on seedlings of Scots pine, Siberian stone pine and Siberian spruce was identified using DNA sequencing. In each of the studied forest nurseries of Middle Siberia the pathogenic microflora are represented by 1-8 genera of micromycetes. The most common phytopathogens detected on 1-5-year old coniferous seedlings were *Phoma* sp., *Alternaria* sp., *Cladosporium* sp., *Lophodermium* sp., *Sclerophoma* sp. and *Typhula* sp. The rest pathogens were less common and their emergence was likely associated with unsuitable cultivation practices.

Conclusions

The study showed that phytopathological monitoring of forest nurseries by molecular genetic analysis is a useful approach for identification of fungal infections in planting material. The identification of pathogens can be helpful in reducing the fungal diseases in forest nurseries of Middle Siberia. It should be recommended to carry out preventive measures against detected phytopathogens with fungicides allowed to be used in forestry. However, the application of chemicals for diseases control is quite uncertain in forest nursery production in Russia. Most of the available fungicides are registered only for agricultural crops. New, effective and eco-friendly fungicides are not introduced yet, which is a serious problem for afforestation. Therefore, considerable research for environmentally safe and easily biodegradable biofungicides should be carried out.

List of abbreviations

- DNA - deoxyribonucleic acid;
- PCR - polymerase chain reaction

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