Environmental Engineering and Management Journal

July 2019, Vol.18, No. 7, 1509-1517
http://www.eemj.icpm.tuiasi.ro/; http://www.eemj.eu

"Gheorghe Asachi" Technical University of Iasi, Romania

FUNGI ASSOCIATED WITH CONIFER SEEDLINGS GROWN IN FOREST NURSERIES UNDER DIFFERENT SYSTEMS

Adam Okorski[1*], Agnieszka Pszczółkowska[1], Alicja Gorzkowska[1], Sylwia Okorska[2], Patryk Głuszek[1]

[1]Department of Entomology, Phytopathology and Molecular Diagnostics, University of Warmia and Mazury in Olsztyn, pl Łódzki 5, 10-727 Olsztyn, Poland
[2]Department of Plant Physiology, Genetics and Biotechnology, University of Warmia and Mazury in Olsztyn, Oczapowskiego 1A, 10–719 Olsztyn, Poland

Abstract

The diversity of fungi colonizing pine and spruce seedlings was analyzed in container and field (bare-root) nurseries in the Region of Warmia and Mazury, Poland. The presence of pathogenic fungi and *Oomycetes* belonging to the genera *Botrytis*, *Cylindrocarpon*, *Fusarium*, *Pestalotia*, *Phoma*, *Phytophthora*, *Pythium* and *Rhizoctonia* was noted in the sampled seedlings. The presence of the pathogenic species *C. destructans* and species belonging to the genera *Fusarium* and *Phytophthora* on the roots and stems of coniferous tree seedlings was also determined with the use of SCAR PCR molecular markers. This method proved to be more accurate than the conventional cultivation method. Conventional mycological analyses revealed that pine and spruce roots were significantly colonized by pathogenic fungi which accounted for 55.5% and 50.14% of all fungi, respectively. Pine stems were more severely infected with pathogenic fungi at 61.5%, whereas spruce stems were colonized by pathogenic fungi in 53.83%. Pathogenic fungi were more abundant on pine and spruce seedlings from the container nursery, whereas a comparison of the species richness index with the Shannon diversity index revealed greater species diversity of fungal communities in samples from field nurseries than in seedlings from container nurseries. The studies showed also the association of fungal communities with a specific nursery, which was probably influenced by the way of cultivation. Based on the research high efficiency of fungal identification by SCAR PCR method was found, this method should be recommended as part of integrated plant protection for wide use, especially in container nursery.

Keywords: forest nurseries, fungal diversity, *Fusarium*, pine, root pathogens, SCAR PCR, spruce

Received: January, 2018; Revised final: April, 2018; Accepted: June, 2018; Published in final edited form: July, 2019

1. Introduction

Afforestation projects and sustainable conversion of farmland to non-agricultural uses require healthy and high-quality seedlings (Kwaśna and Bateman, 2009). Container nurseries provide optimal conditions for the growth and development of forest seedlings (Szabla, 2009). In systems where the root system is containerized, seedlings are grown in substrates with favorable physicochemical properties. Soil moisture, the physiological condition of seedlings, their demand for nutrients and nutrient leaching can be carefully monitored. Controlled mycorrhization and biological and chemical protective treatments are applied, and pests and pathogens are actively eliminated (Buraczyk and Szeligowski, 2008; Kormanek et al., 2013; Okorski et al., 2014; Szabla 2009). Biological diversity in a forest ecosystem determines the health of trees because the availability of chemical protection products recommended for forest management is decreasing steadily (Okorski et al., 2015b).

Seedling blight and root rot, which develop several weeks after germination, are ubiquitous

*Author to whom all correspondence should be addressed: e-mail: adam.okorski@uwm.edu.pl; Phone:+48895233411

diseases in forest nurseries. They are caused by soil pathogens as well as pathogens without host specialization which are disseminated by seeds. Those pathogens contribute to rapid decomposition of plant tissue, and they prevent seed germination and seedling emergence (Sutherland et al., 2002).

Fungi which cause the greatest losses in nurseries include *Alternaria*, *Cylindrocarpon*, *Cylindrocladium*, *Fusarium*, *Trichothecium* and *Rhizoctonia*, most of which are identified in the conidial stage (Sutherland et al., 2002; Verma and Yadav, 2018), as well as *Oomycetes* of the genera *Pythium* and *Phytophthora* which are disseminated with seeds and in soil by zoospores, oospores and chlamydospores (Lilja et al., 2010). Those dangerous pathogens with a wide host range are transferred to forests with seedlings from infected nurseries, and they colonize both annual and perennial plants (Orlikowski et. al., 2011). Environmental factors play an important role in the spread of disease and the transmission of pathogens in conventional forest nurseries. Forest trees are colonized mostly by fungal pathogens, and infections generally develop at the site of tissue damage.

Unfavorable environmental conditions and abiotic stress contribute to the spread of fungal infections. In container nurseries which aim to maximize their output, the risk of disease is very high despite the use of pathogen-free substrates, healthy seeds and controlled growing conditions (Lilja et al., 2010). In unprotected forest nurseries, seedling blight can damage up to 80% of young plants (Mańka, 1993). Molecular biology methods can be used to facilitate the identification of pathogens which cause many diseases (Nevoigt et al., 2010). Pathogenic agents can be detected by PCR in infected plant tissues in the latent stage and directly in soil (Nowakowska et al., 2016b). Pathogens are very difficult to identify under a microscope based on their morphological traits due to similarities in species descriptions, the absence of morphological structures supporting microscopic evaluations, the need to apply specific growing conditions to produce morphological structures or the predominance of fast-growing species in fungal cultures (Garzon et al., 2007).

For this reason, modern diagnostic tools based on DNA markers are indispensable in forest phytopathology (Nowakowska et al., 2016a). The aim of this study was to determine the persistence of fungal pathogens responsible for seedling blight of conifers (pine and spruce) in field (bare-root) and container nurseries.

2. Materials and methods

2.1. Studied localities

The experiment was carried out in four nurseries (two container nurseries and two bare-root nurseries) selected by the Regional Directorate of State Forests in Olsztyn (Poland). Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) H. Karsten) seedlings were sampled for the study one year after sowing.

In the container nursery, pine and spruce seedlings were grown in HIKO V-120 SS (BCC) polypropylene trays measuring 352/216/110 mm (L/W/H) with 120 cm^3 (0.12 l) cells and Roben V EPS polystyrene containers, measuring 650/312/180 mm (L/W/H) with 275 cm^3 (0.275 l) cells. Seedlings were grown on peat substrate made from sphagnum peat moss with a decomposition rate of up to 15% (85 - 87%), perlite with 3-6 mm (12-15%) particle size and milled dolomite added for acid neutralization at 2-4 kg per 1 m^3 of the substrate. In field (bare-root) nurseries, seedlings were grown in accordance with nursery standards, and pine and spruce seedlings were sampled for the experiment in the first year after sowing on similar dates in all nurseries (48 h). Ten spruce seedlings and ten pine seedlings were randomly selected for the experiment. In a laboratory, seedlings were divided into roots and stems which were analyzed separately.

2.2. Mycological analysis of root and stem fungi

Small fragments were cut out from plant tissues selected for the study, they were rinsed in distilled water and disinfected in 50% ethyl alcohol solution for 30 seconds and in 1% sodium hypochlorite (NaOCl) for 30 seconds. The samples were rinsed three times in sterile water and dried, and 2 mm sections were transferred to Petri plates (six sections per plate) containing potato dextrose agar (PDA). The plates were incubated at a temperature of 23°C in darkness for 5 days. The emerging filamentous fungi were transferred to PDA slants and identified based on the available keys and published data (monographs).

2.3. Identification of fungi and Oomycetes by SCAR PCR

DNA was isolated from plant tissues (in three biological replications per sample) with the use of the Invisorb® Spin Plant Mini Kit (Stratec Biomedical). PCR reaction volume was 25 µl, and PCR analyses were carried out with MasterAMP TFL DNA polymerase (Epicentre Technologies) in the Mastercycler Gradient thermocycler (Eppendorf). The applied PCR primers are presented in Table 1. PCR products were separated by electrophoresis in 1.5% agarose gel with ethidium bromide and 1xTBE buffer.

2.4. Analysis of species diversity indices

Species diversity was examined by principal component analysis (PCA) in Canoco vs. 5 software. The database of environmental factors was screened by PCA to evaluate the main trends in species diversity in the analyzed sites. The diversity of endophytic fungi was quantified based on the species richness index and the Shannon diversity index (H′) with the use of the Eq. (1):

Table 1. List of SCAR-based PCR primers for detecting fungal pathogens in forest nurseries

Fungal species/genus	*Primer sequence*	*Amplicon length*	*References*
Cylindrocarpon destructans	CdU3 5' GACGATTCGGGCCGTATCTGTG 3'	500 bp	Seifert et al. (2003)
	CdL1b 5' CAGCGGCGCCCACTAACAAC 3'		
Fusarium	p58SL 5' AGT ATT CTG GCG GGC ATG CCT GT 3'	339 bp	Hue et al. (1999)
	P28SL 5' ACA AAT TAC AAC TCG GGC CCG AGA 3'		
Phytophthora	Yph1F 5' CGACCATKGGTGTGGACTTT 3'	450bp	Schena et al. (2006)
	Yph2R 5' ACGTTCTCMCAGGCGTATCT 3'		

$$H' = -\sum_i Pi \ln(Pi) \quad (1)$$

where: *Pi* is the relative abundance of a species in a given sample. The values of *H'* began from 0 (only one species present with no uncertainty as to the species of each individual) and increased to reveal high uncertainty due to relatively uniform distribution of species.

3. Results and discussion

A total of 375 fungal isolates were obtained from pine roots in both years of the study, including 236 in the first year and 139 in the second year (Table 2). Pine stems were less abundantly colonized by fungi - 211 isolates were obtained in 2013 and 148 in 2014. Pathogenic fungi colonizing pine seedlings belonged to the genera *Botrytis*, *Cylindrocarpon*, *Fusarium*, *Pestalotia*, *Phoma*, *Phytophthora*, *Pythium* and *Rhizoctonia*.

Pathogenic isolates accounted for 55.5% of fungi (208 isolates) colonizing roots and 61.5% of fungi (221 isolates) colonizing stems of pine seedlings. Pathogenic fungi isolated from pine roots represented 50% of all isolates in bare-root nurseries and 62% of all isolates in container nurseries. Pine stems were more severely infected by pathogenic fungi. Pathogenic species accounted for 55% of total isolates in field nurseries and 61% in container nurseries (Table 2).

A comparison of the species diversity of fungi isolated from pine roots and stems in container nurseries and field (bare-root) nurseries revealed that pine roots in field nurseries were colonized by 35 taxa and non-sporulating fungi. Pine roots in container nurseries were colonized by 18 taxa and non-sporulating fungi (Table 2). A total of 349 fungal isolates were obtained from spruce roots, including 150 in the first year (2013) and 199 in the second year (2014) of the study (Table 3).

Table 2. Fungi isolated from pine seedlings in four nurseries in both years of the study (2013-2014)

Fungal species	*Abbrev.*	*field nursery A*				*field nursery B*				*container nursery A*				*container nursery B*			
		root		*stem*		*root*		*stem*		*root*		*stem*		*root*		*stem*	
		2013	*2014*	*2013*	*2014*	*2013*	*2014*	*2013*	*2014*	*2013*	*2014*	*2013*	*2014*	*2013*	*2014*	*2013*	*2014*
Alternaria alternata	A_alt*	11		7	3	14	3	5	3	3	2	3		3	2	3	5
Aureobasidium bolleyi	A_boll	1	1	4	1		2	1	1	3							
Botrytis cinerea	B_cin					7		6	2			4	2			3	5
Cladosporium cladosporioides	Cl_cl	5	1	4	6	5	1		1	6		4		2	3		
Cylindrocarpon destructans	Cy_des	4		8	2	4			3	3	2	2	1	3	3		2
Cylindrocarpon didymum	Cy_di			1	1				1		2			3	4	2	
Cylindrocarpon obtusisporum	Cy_ob	5			1	1		3		1	4		4		3		
Cylindrocarpon sp.	Cy_	4				1		1		1		2	3		2		
Eladia saccula	El_sa	1															
Endothia sp.	En_		4					1									
Epicoccum purpurascens	Ep_pu	2												1			
Fusarium avenaceum	F_av	3	1	8	2	5	3			2		3		8	12	16	17
Fusarium oxysporum	F_ox	6		8	4	4	1	10	4	5	15	6	11	2	1		
Fusarium tricinctum	F_tr				2			1	2	2	4	6	10	1		3	
Fusarium sporotrichioides	F_sp				1		1	1	1	1				1		3	
Gliocladium catenulatum	G_ca	2					2	2				11					
Gliocladium fimbriatum	G_fi		1			1			3								
Mucor circinelloides	Mu_ci	2	2					2									

Mucor elegans	Mu_el	3			1													
Mortierella alpina	Mo_al			4	1							1						
Mortierella candelabrum	Mo_ca		1				1											
Mortierella elongata	Mo_el						1											
Mortierella gracilis	Mo_gr	1			1	2		1										
Mortierella pusilla	Mo_pu				1													
Papulaspora sp.	Pa	3						3										
Penicillium sp.	Pen_	2	2	4		1	1	1	2	6	2	4	2	6	1	2	6	
Pestalotia sp.	Pes_	10	2	12	3	1		2	1									
Phoma sp.	Ph_	3	3	2		2		3	4	2				2				
Phytophthora cactorum	Phy_ca	3	1	1			3											
Phytophthora plurivora	Phy_pl	4	2	7	1													
Phytophthora sp.	Phy_	5	3	6	1	3	3		2									
Pyrenochaeta mackinnonii	Pyr_ma							3		3								
Pythium ultimum	Pyt_ul	3				2			2									
Pythium debaryanum	Pyt_de		1			2	1		2									
Pythium sp.	Pyt_	2	2		2	1	2	1										
Rhizoctonia solani	R_so	2	4			5	6											
Scopulariopsis brevicaulis	S_br		1															
Trichoderma hamatum	T_ham	1		1	1	1				1		1						
Trichoderma harzianum	T_har	2	2		2			1	1	1								
Trichoderma koningii	T_kon					2	2			2								
mycelia sterilia	mc	2	5	4	3	2	2	2	3	3	3		1	1		1	1	
Sum		92	39	81	40	66	35	50	38	45	34	47	34	33	31	33	36	

The abbreviations refers to Fig.1 and 2

Table 3. Fungi isolated from spruce seedlings in four nurseries in both years of the study (2013-2014)

Fungal species	***Abbrev.***	***field nursery A***				***field nursery B***				***container nursery A***				***container nursery B***			
		root		***stem***		***root***		***stem***		***root***		***stem***		***root***		***stem***	
		2013	***2014***	***2013***	***2014***	***2013***	***2014***	***2013***	***2014***	***2013***	***2014***	***2013***	***2014***	***2013***	***2014***	***2013***	***2014***
Alternaria alternata	A_alt	1	3	1	5	2	6	3	10	3	4	5	2	6	1	3	3
Aspergillus niger	A_ni		2		2	1		2	1								
Aureobasidium bolleyi	A_boll	1			4		1	2	2		2		3		2		
Botrytis cinerea	B_cin			2					2	2	1				3		2
Cladosporium cladosporioides	Cl_cl	5	4	2	3	5	6	3	5	2	1	8	2	3	5	4	2
Cylindrocarpon destructans	Cy_des	1	2	1			5		5					1	2		
Cylindrocarpon didymum	Cy_di	1	5	2	3		6	2		3	1						
Cylindrocarpon obtusisporum	Cy_ob		1		4												
Cylindrocarpon sp.	Cy_	2	4	1		2	1		2	1		2		2	3		
Eladia saccula	El_sa					1		2	3								
Endothia sp.	En_			2													
Epicoccum purpurascens	Ep_pu	2	5	2	3	1						4					
Fusarium avenaceum	F_av	1	1	3	7					3	3	3	17	9	13	14	25
Fusarium oxysporum	F_ox	3	5	5	8		8	2	19	6	9	13	21	3	4	2	5
Fusarium tricinctum	F_tr	1		2			1	2	2	2							
Fusarium sporotrichioides	F_sp	1	2	1		3		1									
Gliocladium catenulatum	G_ca	1				1		2									
Gliocladium fimbriatum	G_fi	2	1	1			1	1	1								
Humicola fuscoatra	H_fu	1		1													
Humicola grisea	H_gr		2														
Mucor circinelloides	Mu_ci	1		2		1		1									

Mucor elegans	Mu_el					1		2	1								
Mortierella alliacea	Mo_all	1		2		1											
Mortierella alpina	Mo_alp		2	2	1			1									
Mortierella candelabrum	Mo_ca			2		2		1	1								
Mortierella elongata.	Mo_el	1				3											
Mortierella gracilis	Mo_gr		1				1										
Mortierella pusilla	Mo_pu			1		1			1								
Papulaspora sp.	Pa_	2			2												
Penicillium sp.	Pen_	4	1	3	2	3	3	2	5	4	2	3	2	2	3	5	3
Pestalotia sp.	Pes_	2	2	4		1	2		2								
Phoma glomerata (	Ph_gl		1			1											
Phoma sp.	Ph_	2	1		3	2	3	4		2	1	2	4				
Phomopsis sp.	Phom_										2						
Phytophthora sp.	Phy_	1	2			2	3			2	7						
Pyrenochaeta mackinnonii	Pyr_ma								1								
Pythium ultimum	Pyt_ul		1				1	1									
Pythium debaryanum	Pyt_de	1				1	2										
Pythium sp.	Pyt_	1				1	1										
Rhizoctonia solani	R_so	2	6			1	9	1	2								
Scopulariopsis brevicaulis	S_br		1	1		1	1		1								
Trichoderma hamatum	T_ham	1	2				2	1									
Trichoderma harzianum	T_har	2	1	1			1	1									
Trichoderma koningii.	T_kon	1				1											
mycelia sterilia	mc	3	2	2	1	3	5	3	4	2	1	1		2		2	
Sum		48	60	46	48	42	69	40	70	32	34	41	51	28	36	30	40

The abbreviations refers to Fig. 1 and 2

A total of 366 fungal cultures were isolated from spruce stems, of which 157 were isolated in 2013 and 209 in 2014. Similarly to pine seedlings, pathogenic fungi isolated from spruce roots and stems belonged mostly to the genera *Botrytis*, *Cylindrocarpon*, *Fusarium*, *Pestalotia*, *Phoma*, *Phytophthora*, *Pythium* and *Rhizoctonia*. Pathogenic fungi isolated from spruce seedlings accounted for 50.14% (175 isolates) of fungi isolated from spruce roots and 53.83% (297) of fungi isolated from stems. Pathogenic fungi isolated from spruce roots accounted for 43.38% of all isolates in field nurseries and for 62% of all isolates in container nurseries, whereas pathogenic fungi isolated from spruce stems represented 42.6% and 68% of all fungi isolated in field and container nurseries, respectively (Table 3). A total of 43 fungal taxa were isolated from spruce roots, including 41 in field nurseries and 15 in container nurseries. Spruce stems were characterized by lower fungal diversity, and the isolated fungi represented 36 species and genera. The stems of spruce seedlings from field nurseries were colonized by 36 taxa, whereas the stems of seedlings from container nurseries - by only 11 taxa.

The presence of fungal species causing seedling blight and root rot, represented by the genera *Cylidrocarpon*, *Fusarium*, *Pestalotia*, *Pythium*, *Phytophthora* and *Rhizoctonia*, was noted in container nurseries (Stewart et al., 2006). In Canada, root rot in forest nurseries is most frequently caused by *Cylindrocarpon destructans* and *Cylindrocladium floridanum*. Both fungal species contributed to the loss of seedlings in nurseries (40-50%) and forest sites (30-40%) (Hamelin et al., 1996). Dumroese and James (2005) identified fungi of the genera *Pythium*, *Fusarium* and *Cylindrocarpon* across the entire area of container nurseries in the Pacific Northwest, whereas *Phytophthora* fungi were persistent on out-planted seedling roots. The similarities and differences between the examined fungal communities colonizing pine roots and stems from the analyzed nurseries are presented in a graphic projection of PCA results (Fig. 1A and 1B). Root fungi were characterized by PCA which revealed that *F. tricinctum* and *F. oxysporum* were the dominant species in container nursery A in both years of the study (vector length) (Fig. 1A). Communities of root fungi in field nursery B (2014), field nursery A (2014) and container nursery B (2013, 2014) were similar (Fig. 1A) with a high percentage of *F. avenaceum* and other non-sporulating fungal species of the genus *Fusarium* (Fig. 1A). Pine roots from field nurseries A (2013) and B (2013) were characterized by significant species richness with a high percentage of *Pythium ultimum* and species of the genus *Phytophthora* (Fig. 1A). In pine roots, PCA revealed the species specificity of fungal communities colonizing seedlings from container nursery B, with a predominance of *F. avenaceum* and other species of the genus *Fusarium* (Fig. 1B). *F. tricinctum* was the dominant species in container nursery A.

Spruce seedlings grown in field nursery A were characterized by high similarity of root fungal communities with a predominance of *F. oxysporum* in 2014 and members of the genera *Phytophthora* and

Pestalotia in 2013 (Fig. 1C). Principal component analysis revealed high similarities in fungal communities colonizing spruce roots with a clear dominance of *F. avenaceum* in both container nurseries (Fig. 1C). In 2013, fungal communities colonizing spruce roots in container nursery B were characterized by a high percentage of antagonistic species of *Trichoderma*, *Gliocladium* and *Mortierella* accompanied by pathogenic fungi of the genera *Fusarium* and *Phytophthora* (Fig. 1C).

The dominant fungal species colonizing spruce stems was *F. avenaceum* in container nursery B and *F. oxysporum* in container nursery A (2014) (Fig. 1D). In field nursery B, a significant percentage of *Fusarium* sp., *Pythium* and the antagonistic species *Trichoderma harzianum* was noted in 2013, and a high proportion of *Cylindrocarpon destructans* was observed in 2014. Similar results were noted in field nursery A in the first year of the study. In the second year, the stems of spruce seedlings from field nursery A were colonized mainly by *F. tricinctum* (Fig. 1D). The values of the species richness index and the Shannon diversity index were high in all samples (spruce and pine) from bare-root nurseries (Fig. 2A, 2B, 2C and 2D). The roots and stems of spruce and pine seedlings from container nurseries were characterized by low values of the species richness index and the Shannon diversity index (Fig. 2A, 2B, 2C and 2D).

The PCA analysis was used in many works regarding to the definition of a fungal community isolated from different hosts or stands (Klamer and Hedlund, 2004; Jankowiak et al., 2016; Stenström et al., 2014). For example, Newton and Haigh (1998) studied the relationship between ectomycorrhizae and their host in Great Britain. In the presented study, the predominance of *F. tricinctum*, *F. oxysporum* and *F. avenaceum* in the fungal communities colonizing the roots and stems of spruce and pine seedlings grown in container nurseries suggests that those pathogens spread via trays, water and other channels.

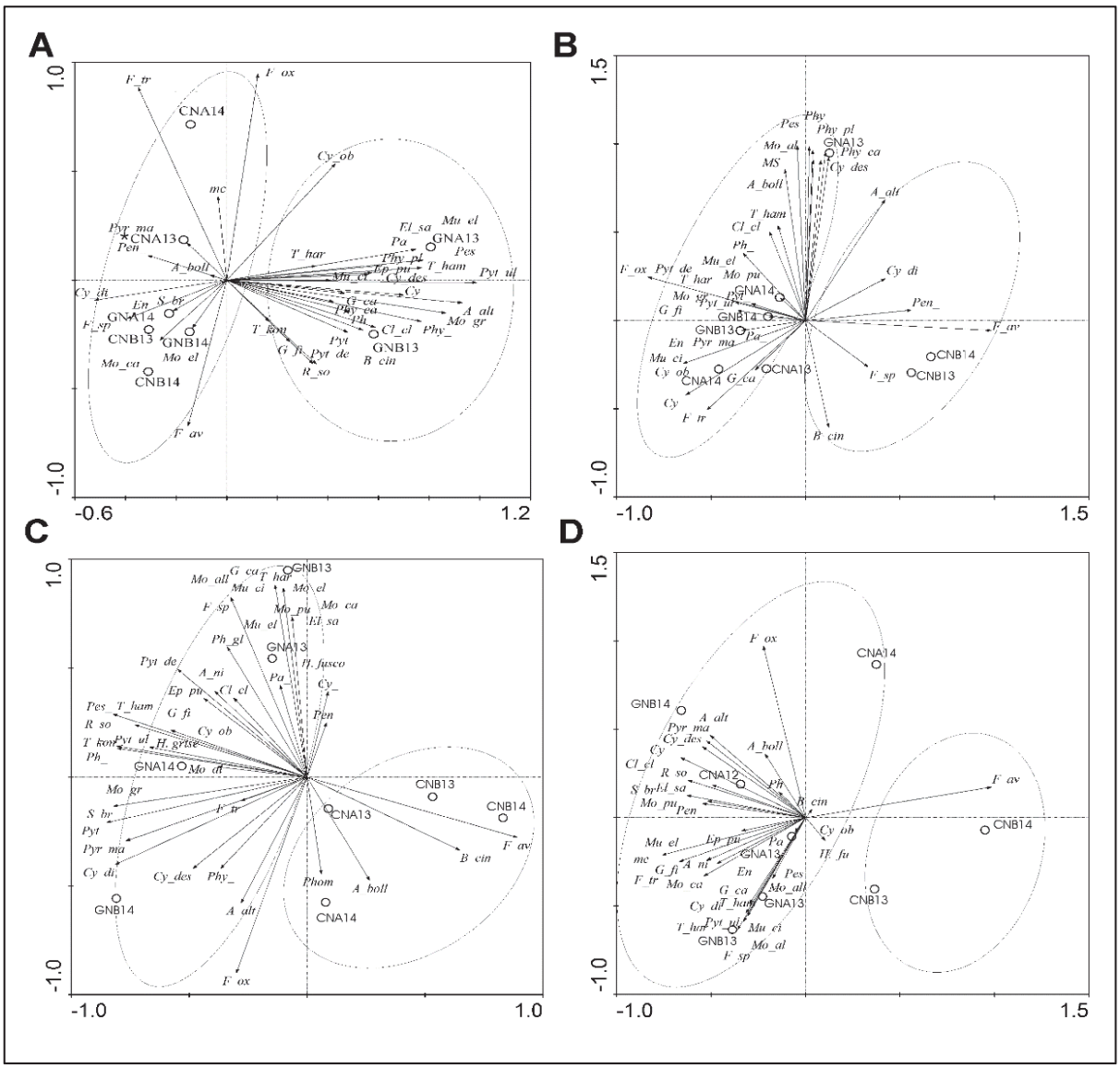

Fig. 1. PCA analysis of fungi colonizing the roots (A, C) and stems (B, D) of pine (A, B) and spruce (C, D) seedlings (*CNA- container nursery A, CNB- container nursery B, GNA- field nursery A, GNB- field nursery B; 13- samples from 2013 year, 14- samples from 2014 year)

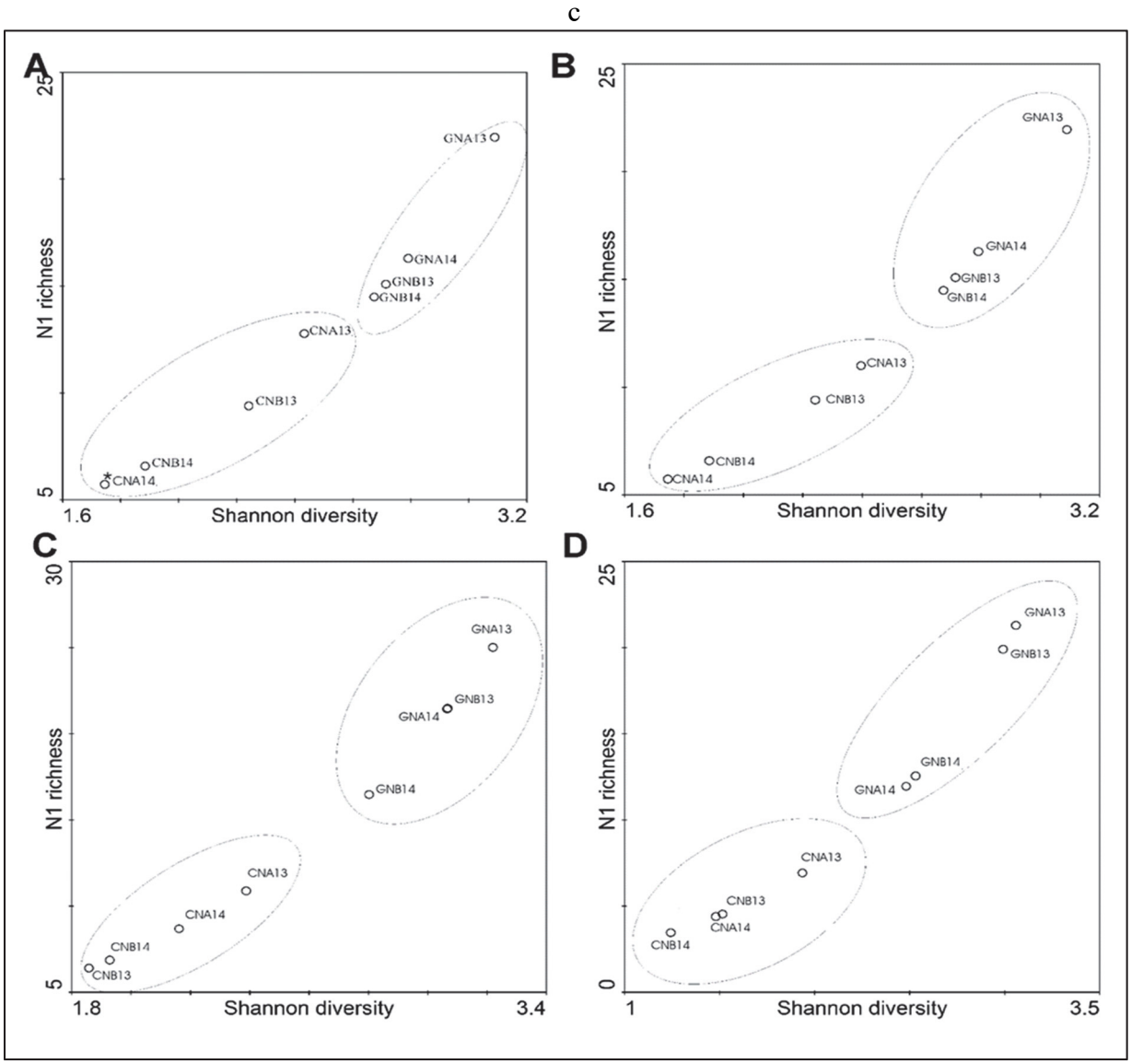

Fig. 2. Species richness index and Shannon diversity index H' of fungi colonizing the roots (A, C) and stems (B, D) of pine (A, B) and spruce (C, D) seedlings (*CNA- container nursery A, CNB- container nursery B, GNA- field nursery A, GNB- field nursery B; 13- samples from 2013 year, 14- samples from 2014 year)

Nursery cultural practices influence fungal community structure colonizing the seedlings. For instance, the different nurseries may vary sowing densities and amount of fertilizer applied (Stenström et al., 2014). These differences might be enough to influence fungal growth in the seedlings. In our study we found that the samples of spruce and pine show some similarity are coming from the same nursery; this indicates the strong influence that the nursery effect has on the fungal community composition in the roots of seedlings.

Low levels of species diversity and species richness were noted, which is characteristic of container nurseries. In forest ecosystems, species diversity is increasingly often analyzed with the use of direct sequencing and NGS methods which are highly reliable but very expensive (Baldrian et al. 2012; Buée et al. 2009; Menkis et al. 2006). New diseases in forest nurseries and forest production have been noted in recent years (Gaffuri et al. 2015; Okorski et al. 2015a; Wit et al. 2015), which indicates that high-output forest nurseries and the spread of new species contribute to the emergence of new pathogens. The new pathogen which has been noted in this study is *F. avenaceum*, a species responsible for strong symptoms of beech disease in gangrenous diseases in previous studies (Okorski et al. 2015a). In a study evaluating the risk of invasive species in forest ecosystems, Santini et al. (2013) classified Poland as a country with a low number of new invasive species, but noted that due to the isolation of the Polish economy in the 20th century, the relevant risk is highly likely to increase in the present century. The introduction of invasive species to container nurseries can lead to high losses in the absence of competitive species in the substrate. For this reason, container nurseries should mycorrhize seedlings (Kuc and Aleksandrowicz-Trzcińska 2013) and apply biological protection products that are

certified for use in forest ecosystems (Okorski et al. 2014). The risk of invasive species in container nurseries can be minimized by isolating infected seedlings and analyzing the substrate (peat), seeds and water used for irrigation (which is often drawn from open reservoirs) for the presence of pathogenic species or genera which can cause significant losses in production. The presence of pathogenic fungi of the genera *Fusarium* and *Cylindrocarpon* and *Oomycetes* of the genus *Phytophthora* in plant tissues was determined by SCAR PCR, but they were not identified in conventional mycological analyses, which indicates that SCAR PCR is a much more reliable diagnostic method (Table 4).

Table 4. SCAR-PCR analysis of pine and spruce seedlings infected by *Fusarium*, *Cylindrocarpon destructans* and *Phytophthora* in four nurseries in both years of the study (2012-2013)

Nursery method	***Plant species***	***N***	***Organ***	***Fusarium***	***Phytophthora***	***Cylindrocarpon destructans***
Field nursery A	pine	9	root	+*/+**	-/+	-/+
		9	stem	+/+	+/-	+/-
	spruce	9	root	+/-	+/+	+/+
		9	stem	+/-	-/-	-/-
Field nursery B	pine	9	root	+/-	+/+	+/+
		9	stem	+/-	-/-	-/-
	spruce	9	root	+/-	+/+	+/+
		9	stem	-/-	-/-	-/-
Container nursery A	pine	9	root	+/+	-/-	+
		9	stem	+/+	-/-	-/-
	spruce	9	root	+/+	-/-	+/-
		9	stem	-/-	-/-	-/-
Container nursery B	pine	9	root	+	-/-	+
		9	stem	+	-/-	-/-
	spruce	9	root	+	-/-	-/-
		9	stem	+	-/-	-/-

**- 2012 year, **- 2013 year*

Fusarium species were identified in most samples from container nurseries, whereas *Phytophthora* species were noted only in seedlings from field nurseries (Table 4). Today it is possible to detect the disease in planting material using a DNA based test. One benefit of this test is its higher sensitivity compared to the traditional isolation (Lilja et al., 2010). Examination of seedlings by PCR should be an important element of disease prevention in container nurseries because sick seedlings may look green and healthy, the problem may increase if seedlings are used for planting immediately after storage in cold stores in Winter, when there are no visible lesions, due to the short time between lifting the cuttings from the cold store and planting into crops. Unfortunately in Poland, the prevalence of invasive species is controlled to a limited extent due to a small number of research centers which monitor such threats. Forest nurseries have to conduct routine inspections with the involvement of all available methods to minimize the spread of invasive species. Practical application of the molecular diagnostics method in forestry nursery in accordance with the dispositions of the European Commission (Directive No 2009/128/WE dated on October 21, 2009 and the Regulation No 546/2011 dated on June 10, 2011 concerning the integrated plant protection against pests), which refer to the use of integrated pest management. However, this requires expansion of research infrastructure and greater awareness on the part of people involved in practice (forestry).

4. Conclusions

In conclusion, our findings suggest that container nurseries were characterized by lower species diversity of fungi colonizing conifer roots and stems in comparison with field (bare-root) nurseries. Fungal pathogens responsible for seedling blight and root rot, representing the genera *Botrytis*, *Cylindrocarpon*, *Fusarium*, *Pestalotia*, *Phoma*, *Phytophthora*, *Pythium* and *Rhizoctonia*, were identified in container and field nurseries. The molecular method with SCAR PCR markers was a highly effective tool for identifying fungal pathogens on pine and spruce seedlings.

Acknowledgements

This study was funded by the Polish Ministry of Science and Higher Education of Poland as the part of statutory activities.

References

Baldrian P., Kolařík M., Stursová M., Kopecký J., Valášková V., (2012), Active and total microbial communities in forest soil are largely different and highly stratified during decomposition, *Multidisciplinary Journal of Microbial Ecology*, **78**, 248-258.

Buée M., Reich M., Murat C., Morin E., Nilsson R.H., (2009), 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity, *New Phytolgist*, **184**, 449-456.

Buraczyk W., Szeligowski H., (2008), The impact of soil's textural group and moisture on the growth of Scots pine (*Pinus sylvestris* L.) seedlings with containerized root system, *Forest Research Papers*, **69**, 291-297.

Dumroese R.K., James R.L., (2005), Root diseases in bareroot and container nurseries of the Pacific Northwest: epidemiology, management, and effects on outplanting performance, *New Forests*, **30**, 185-202.

Gaffuri F., Maresi G., Pedrazzoli F., Longa C.M.O., Boriani M., Molinari M., Tantardini A., (2015), *Colletotrichum acutatum* associated with *Dryocosmus kuriphilus* galls on *Castanea sativa*, *Forest Pathology*, **45**, 169-171.

Garzon C.D., Ya´nez J.M., Moorman G.W., (2007), *Pythium cryptoirregulare*, a new species within the *P. irregulare* complex, *Mycologia*, **99**, 291-301.

Hamelin R.C., Be Rube P., Gignac M., Bourassa M., (1996), Identification of root rot fungi in nursery by nested multiplex PCR, *Applied Environmental Microbiology*, **62**, 4026-4031.

Hue F-X., Huerre M., Rouffault M.A., de Bievre C., (1999), Specific detection of *Fusarium* species in blood and tissues by a PCR technique, *Journal of Clinical Microbiology*, **37**, 2434-2438.

Jankowiak R., Bilański P., Paluch J., Kołodziej Z., (2016), Fungi associated with dieback of *Abies alba* seedlings in naturally regenerating forest ecosystems, *Fungal Ecology*, **24**, 61-69.

Klamer M., Hedlund K., (2004), Fungal diversity in set-aide agricultural soil investigated using terminal-restriction fragment length polymorphism, *Soil Biology & Biochemistry*, **36**, 983-988.

Kormanek M., Banach J., Ryba M., (2013), Influence of substrate compaction in nursery containers on the growth of scots pine (*Pinus sylvestris* L.) seedlings, *Forest Research Papers*, **74**, 307-314.

Kuc T., Aleksandrowicz-Trzcińska M., (2013), Artificial mycorrhization and soil application of fungicides to pedunculate oak seedlings. I. Mycorrhizal colonization and growth of container-grown seedlings in the nursery, *Sylwan*, **156**, 765-775.

Kwaśna H., Bateman G.L., (2009), Microbial communities in roots of *Pinus sylvestris* seedlings with damping-off symptoms in two forest nurseries as determined by ITS1/2 rDNA sequencing, *Forest Pathology*, **39**, 239-248.

Lilja A., Poteri M., Petäistö R.-L., Rikala R., Kurkela T., Kasanen R., (2010), Fungal diseases in forest nurseries in Finland, *Silva Fennica*, **44**, 525-545.

Mańka M. (1993), Communities of fungi from roots and rhizosphere of yew (*Taxus baccata* L.) seedlings and their effect on the pathogenic root fungus *Cylindrocarpon destructans* (Zinss) Scholten, *Phytopathologia Polonica*, **5**, 51-58.

Menkis A., Vasiliauskas R., Taylor A.F.S., Stenström E., Stenlid J., Finlay R., (2006), Fungi in decayed roots of conifer seedlings in forest nurseries, afforested clear-cuts and abandoned farmland, *Plant Pathology,* **55**, 117-129.

Newton A.C., Haigh J.M., (1998), Diversity of ectomycorrhizal fungi in Britain: a test of the species area relationship and the role of host specificity, *New Phytologist*, **138**, 619-627.

Nevoigt F., Oszako T., Nowakowska J.A., (2010), Monitoring of diseases caused by pathogenic oomycetes with the help of DNA analysis. *Sylwan*, **154**, 450-455.

Nowakowska J.A., Malewski T., Tereba A., Oszako T., (2016a), Rapid diagnosis of pathogenic *Phytophthora* species in soil by Real-Time PCR, *Forest Pathology*, **47**, 1-4.

Nowakowska J.A., Malewski T., Tereba A., Borys M., Oszako T., (2016b), Molecular diagnostic of chosen pathogens from *Phytophthora* genus within Integrated Plant Management (IPM), *Sylwan*, **160**, 365-370.

Okorski A., Oszako T., Nowakowska J.A., Pszczółkowska A., (2014), The possibilities of biologically protecting plants against diseases in nurseries, with special consideration of *Oomycetes* and *Fusarium* fungi, *Forest Research Papers*, **75**, 301-321.

Okorski A., Pszczółkowska A., Okorska S., Fordoński G., (2015a), First report of *Fagus sylvatica* infection by *Fusarium avenaceum* in forest container nurseries in north-eastern Poland, *Plant Disease*, **99**, 420, doi.org/10.1094/PDIS-09-14-0899-PDN.

Okorski A., Pszczółkowska A., Oszako T., Nowakowska J.A., (2015b), Current possibilities and prospects of using fungicides in forestry, *Forest Research Papers*, **76**, 191-206.

Orlikowski L.B., Oszako T., Ptaszek M., (2011), Menace of forest nurseries by species of the *Phytophthora* genus, *Sylwan*, **155**, 322-329.

Santini A., Ghelardini L., De Pace C., Desprez-Loustau M.L., Capretti P., Chandelier A., Cech T., Chira D., Diamandis S., Gaitniekis T., Hantula J., Holdenrieder O., Jankovsky L., Jung T., Jurc D., Kirisits T., Kunca A., Lygis V., Malecka M., Marcais B., Schmitz S., Schumacher J., Solheim H., Solla A., Szabò I., Tsopelas P., Vannini A., Vettraino A.M., Webber J., Woodward S., Stenlid J., (2013), Biogeographical patterns and determinants of invasion by forest pathogens in Europe, *New Phytologist*, **197**, 238-250.

Schena L., Hughes K.J.D., Cooke D.E.L., (2006), Detection and quantification of *P. ramorum, P. kernoviae, P. citricola* and *P. quercina* in symptomatic leaves by Multiplex Real-Time PCR, *Molecular Plant Pathology*, **7**, 365-379.

Seifert K.A., McMullen C.R., Yee D., Reeleder R.D., Dobinson K.F., (2003), Molecular differentiation and detection of ginseng-adapted isolates of the root rot fungus *Cylindrocarpon destructans*, *Phytopathology*, **93**, 1533-1542.

Stenström E., Ndobe E., Jonsson M., Stenlid J., Menkis A., (2014), Root-associated fungi of healthy-looking *Pinus sylvestris* and *Picea abies* seedlings in Swedish forest nurseries, *Scandinavian Journal of Forest Research*, **29,** 12-21.

Stewart J.E., Kim M.S., James R.L., Dumroese R.K., Klopfenstein N.B., (2006), Molecular characterization of *Fusarium oxysporum* and *F. commune* isolates from a conifer nursery, *Phytopathology*, **96**, 1124–1133.

Sutherland J.R., Diekmann M., Berjak P., (2002), Forest tree seed health for germplasm conservation, IPGRI Technical Bulletin*,* No. 6, International Plant Genetic Resources Institute, Rome, Italy, On line at: https://www.bioversityinternational.org/fileadmin/_migrated/uploads/tx_news/Forest_tree_seed_health_for_germplasm_conservation_865.pdf.

Szabla K., (2009), Silvicultural and economic aspects of container−grown seedling production subjected to controlled mycorrhization, *Sylwan*, **153**, 253−259.

Verma J.P., Yadav J., (2018), Implication of microbial consortium on biomass and yield of chickpea under sustainable agriculture, *Environmental Engineering and Management Journal*, **17**, 513-522.

Wit M., Sierota Z., Oszako T., Mierzwa-Mróz E., Wakuliński W., (2015), *Fusarium* spp. on the above-ground organs of dying oaks - a new threat?, *Sylwan*, **159**, 403−410.