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Mycorrhizal Fungal Community of Poplars Growing on Pyrite Tailings Contaminated Site near the River Timok

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Abstract

Background and Purpose: Mycorrhizal fungi are of high importance for functioning of forest ecosystems and they could be used as indicators of environmental stress. The aim of this research was to analyze ectomycorrhizal community structure and to determine root colonization rate with ectomycorrhizal, arbuscular mycorrhizal and endophytic fungi of poplars growing on pyrite tailings contaminated site near the river Timok (Eastern Serbia).

Materials and Methods: Identification of ectomycorrhizal types was performed by combining morphological and anatomical characterization of ectomycorrhizae with molecular identification approach, based on sequencing of the nuclear ITS rRNA region. Also, colonization of poplar roots with ectomycorrhizal, arbuscular mycorrhizal and dark septated endophytic fungi were analysed with intersection method.

Results and Conclusions: Physico-chemical analyses of soil from studied site showed unfavourable water properties of soil, relatively low pH and high content of heavy metals (copper and zinc). In investigated samples only four different ectomycorrhizal fungi were found. To the species level were identified *Thelephora terrestris* and *Tomentella ellisi,* while two types remained unidentified. Type *Thelephora terrestris* made up 89% of all ectomycorrhizal roots on studied site. Consequently total values of Species richness index and Shannon-Weaver diversity index were 0.80 and 0.43, respectively. No structures of arbuscular mycorrhizal fungi were recorded. Unfavourable environmental conditions prevailing on investigated site caused decrease of ectomycorrhizal types diversity. Our findings point out that mycorrhyzal fungal community could be used as an appropriate indicator of environmental changes.

Keywords: ectomycorrhiza, molecular identification, poplars, Timok, pyrite tailing

INTRODUCTION

Mining complex in the vicinity of Bor (Eastern Serbia) represents a considerable source of environmental pollution. Soil from a large area near the river Timok was contaminated by flotation tailing composed mainly of pyrite (FeS_2). Consequently vegetation in this area suffered abiotic stress induced by a low pH, high content of copper and lead, deficiency of soil organic matter and severe deficiency of the available mineral nutrients [1].

Poplars are woody species suitable for phytoremediation purposes, because they can extract or incorporate into their aboveground tissues or stabilize in their root systems numerous contaminants from soil [2, 3]. They are also well adapted to a broad range of climatic conditions and soils, have deep root systems, cycle large amounts of water and grow rapidly producing large amount of biomass [2-4]. Soil microorganisms such as mycorrhizal fungi could have important role in phytoremediation because they can modify bioavailability of heavy metals and/or increase plant growth [5]. Poplars can make functional associations with both ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi [6]. Since these two mycorrhizal forms are known to prefer different climate and soil conditions such as nutrient content, pH and C/N ratio [7], dual colonization enables poplars to have broader ecological valence.

Mycorrhizal fungi facilitate the establishment and survival of vegetation under stress condition providing nutrients and water otherwise not accessible for plants [8]. With a net of their hyphae they can stabilize the tailing material and improve soil structure while with compounds produced by the extraradical mycelium can accumulate or chelate heavy metals [9]. In order to be efficient for use in phytoremediation techniques, mycorrhizal fungus has to satisfy two important conditions: tolerance to high concentration of heavy metals in soil and good functional compatibility with a plant used in phytoremediation [10].

Functional compatibility and stress tolerance in mycorrhiza are species specific and depend on both partner [11] therefore the information on the ECM community structure can provide valuable information about physiology of forest trees and functioning of forest ecosystems [12].

The aim of this research was to analyze ectomycorrhizal community structure and to determine root colonization rate with ectomycorrhizal, arbuscular mycorrhizal and endophytic fungi of poplars growing on pyrite tailings contaminated site near the river Timok. This information could be helpful in further research on creating inoculum for afforestation of sites contaminated with pyrite tailings.

MATERIAL AND METHODS

Physico-Chemical Properties of Soil from the Pyrite Tailings Contaminated Site Timok

Physical and chemical properties were determined in the surface layer of the soil (up to 30 cm). The following soil characteristics were analyzed: particle size distribution (%) by the international B-pipette method with the preparation in sodium pyrophosphate [13], determination of soil textural classes based on particle size distribution by using Atteberg classification, $CaCO_3$ percentage (%) was measured volumetrically by using Scheibler's calcimeter [14] and pH in H_2O and KCl were determined by electrometric method with combined electrode on Radiometer pH meter. Concentrations of heavy metals in soil were determined with Atomic Absorption Spectrophotometer (VARIAN AAS 240 FS). All analysis were performed in the laboratory of Soil Science in the Institute of Lowland Forestry and Environment in Novi Sad.

Site

Mycorrhizal fine roots were isolated from soil samples collected in the river land of the river Timok (N 44º00'29.96'', E 22º21'54.48'', 228 m a.s.l.) located about 20 km from Zaječar town, in Eastern Serbia. Site was covered with naturally grown cca 40 years old poplar trees

(*Populus alba* L., *P. nigra* L., *P. tremula* L. and their hybrids) mixed with *Amorpha fruticosa* L., *Betula pendula* Roth and *Alnus glutinosa* L. Climate is temperate continental with the average annual precipitation in the area of 581.4 mm. Average temperature of the air in January is -0.2ºC, in July 22.4ºC, while the average yearly temperature is 11°C (The Republic Hydrometeorological Service of Serbia http://www.hidmet.gov.rs/).

Sampling

Five mature white poplar trees were randomly selected for sampling. For ECM community analysis two soil samples per tree were taken in July 2010, at a distance of about 1m from the tree trunk. A soil core of 274 ml volume and 18 cm deep was used for taking standardized samples [15]. In total, ten soil core samples were collected and kept stored at 4ºC for up to three months. Prior to mycorrhizal analysis each soil core was submerged in cold tap water to loosen the soil structure. Roots were carefully washed from soil and vital ECM root tips were separated from old, nonturgescent and nonmycorrhizal (ONN) root tips in water under a dissecting microscope. For the determination of root length colonization with ECM, AM and endophytic (END) fungi five additional soil samples per plant (in total 25) were taken in August 2011 using the same sampling approach.

Identification of Ectomycorrhizae

ECM types were identified by combining morphological and anatomical approach with molecular methods performed at the laboratory of the Department of Forest Physiology and Genetics in Slovenian Forestry Institute in Ljubljana, Slovenia.

Morphological and anatomical characteristics of each ECM type were assessed by a binocular Olympus SZX 12 (light source Olympus Highlight 3100, daylight filter) and microscope Olympus BX 51 (magnification 100-2000 x) following methodology proposed by Agerer [16] and Kraigher [17], and ECM descriptions published in Agerer [18], Agerer *et al*. [19], and Agerer and Rambold [20]. All fine root tips categories were manually counted under the stereomicroscope. Based on the presence and abundance of emanating elements, ECM types were also classified into the exploration types proposed by Agerer [21].

Molecular identification was based on nucleotide sequencing of ITS (Internal Transcribed Spacer) regions in nuclear ribosomal DNA. This molecular marker is considered as the best for fungi identification [22]. After DNA extraction from 5-20 root tips with a PlantDNeasy Mini Kit (Qiagen, Hilden, Germany) from each ECM type ITS region was amplified with ITS 1f and ITS 4 primer pair [23]. DNA fragments were separated in and excised from agarose gel and purified with Wizard® SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI, USA). Sequencing was performed commercially at Macrogen Inc. (Seoul, Rep. of Korea). Species, genus or family of ectomycorrhizal fungi were determined by comparing sequences to the ones deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/index.html) and Unite databases [24].

Root Colonization

Before evaluation, poplar's fine roots from 5 soil samples were isolated from soil, separated from the roots of herbaceous species by means of visual inspection and jointed together in one sample. Extracted roots were gently washed and cleared in 10% potassium hydroxide and stained with Trypan blue in lactoglycerol according to Kormanik and McGraw [25] and Karliński *et al.* [26]. Colonization of poplar roots by ECM, AM and END fungi was evaluated using the intersection method by McGonigle *et al.* [27] modified by Karliński *et al.* [26] at 200× magnification. A minimum of 200 line intersections per subsample (microscopic slide) were scored for the presence of AM structures (hyphae, vesicles, arbuscules, and coils), ECM or END fungi. Cross section without fungal structures was counted as "empty root". The results are presented as a percentage of root length colonized i.e. partition of number of particular fungal structures in total number of cross sections.

Data Analysis

Diversity indices (Shannon-Weaver index, Species richness index, Evenness, Equitability and Berger-Parker index) were calculated per sample and per site in the way that ECM community data were pooled after formulas given by Atlas and Bartha [28]:

- Species richness (d): $d = (S-1) / \log(10)N$,

where S - number of ECM types, N - number of all mycorrhizal tips;

- Shannon Weaver diversity index (H): $H = C/N (N \cdot \log N - \sum n_i \log n_i)$,

where C - 2,3, N - number of all mycorrhizal tips, n_i - number of mycorrhizal tips of individual ECM type;

- Evenness (e); $e = H / \log S$,

where H - Shannon Weaver diversity index, S - number of ECM types;

- Equitability (J): $J = H / H_{max}$,

where H - Shannon Weaver diversity index, H_{max} - theretical maximal H assuming each ECM type was represented with one mycorrhizal tip;

- Berger-Parker evenness index (BP): $BP = 1 - (N_{max} / N)$,

where N_{max} = number of mycorrhizal tips of the most frequent ECM type, N=number of all mycorrhizal tips.

Relative abundance of ECM types was calculated as a ratio between the tips number of individual ECM type and total number of ECM tips.

RESULTS

Physical and chemical analysis of examined soil samples from pyrite tailings contaminated site showed high content of total sand (91.4%), moderately acid pH (4.91) (according to Dugalić and Galić [29]) and very low concentration of nitrogen (0.06%) (Table 1). According to its granulometric content soil can be classified in the sand texture class. Also, it belongs to the group of technogenous soils, type deposol [30]. Origin of this soil type is related to the undeveloped

TABLE 1. Granulometric composition and some chemical properties of soil from Timok site

Coarse sand (%)	Fine sand (%)	Dust (%)	Clay (%)	Texture class	pH in H_2O	pH in KCl	$CaCO_3$	Carbon	Nitrogen	C/N
8.2	83.2	3.0	5.6	Sand	4.91	4.74	1.67	4.71	0.06	78.34

TABLE 2. Concentrations of heavy metals in soil from Timok site (with their maximum amounts allowed in the soil according to the National legislation)

Heavy metal	Concentration (ppm)	Maximum amount allowed in the soil ($mg \cdot kg^{-1}$)
Cr	28.3	100
Ni	14.4	50
Cd	3.3*	3.0
Pb	83.4	100
Cu	896.9*	100
Mn	147.5	/
Fe	58141.21	/
Zn	413.9*	300

* Values higher than maximum allowed by National legislation

alluvial soil (fluvisol) but subsequently influenced by the spilling of pyrite tailings and Fe_2S deposition causing lower pH and contamination with heavy metals. Comparison of heavy metals content in soil from studied site with the National legislation limits [31] has shown that concentration of copper was almost 9x higher than its maximum allowed amount, while zinc was increased and cadmium was slightly above the allowed amount (Table 2).

Analyzing 34042 fine roots on Timok site four ECM types were recorded, while values of Species richness index and Shannon-Weaver index were 0.80 and 0.43, respectively. In average 1.4 ECM types, 550.6 vital mycorrhizal root tips and 3404 all fine roots were recorded per soil sample. Consequently, average values of diversity indices were extremely low (Table 3). In total, at site Timok four ECM types were recorded. Two of them were identified to the species level: *Thelephora terrestris* and *Tomentella ellisii*, while two ECM types remained unidentified (Table 4). *Thelephora terrestris* made up 89% of all ECM roots (Figure 1) and consequently medium distance exploration type made up almost 90% of all ECM roots on this site (data were not shown). Although both fungal groups, Ascomycota and Basidiomycota had two members, Basidiomycota was much more abundant and made up 89.4% of all ECM roots (data not shown). In examined poplar roots no AM fungal structures were observed, while ECM and END fungi colonized 18.19% and 2.23% of root length, respectively (Table 5).

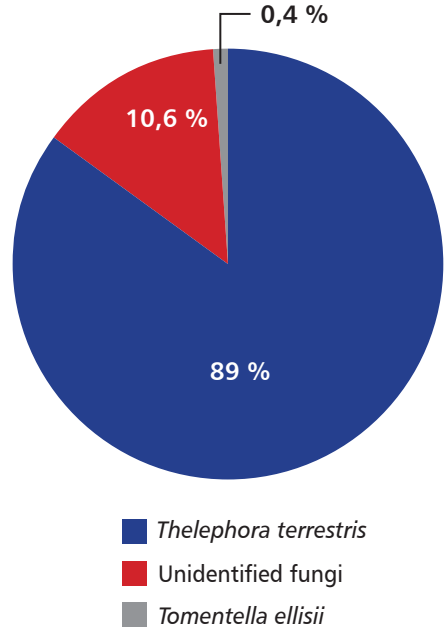

FIGURE 1. Ectomycorrhizal fungi community structure of poplars from Timok site

TABLE 3. Total values and average values per sample (±standard error) for number of ectomycorrhizal (ECM) types, number of vital ECM roots, old, nonturgescent and nonmycorrhizal roots, number of all roots, percentage of vital mycorrhizal roots and diversity indices on investigated site Timok (based on 10 samples)

Examined parameters	Total value for site	Average value per sample
Number of ECM types	4	1.3±0.16
Number of vital mycorrhizal roots		550.6±184.6
Old nonturgescent and nonmycorrhizal roots		2853.6±892.2
Number of all roots		3404.2±946.4
Percentage of vital mycorrhizal roots		19.7±5.0
Species richness index (d)	0.80	0.144±0.06
Shannon-Weaver index	0.43	0.138±0.07
Equitability (J)	0.31	0.199±0.10
Eveness (e)	0.71	0.461±0.23
Berger-Parker index	0.11	0.045±0.03

TABLE 4. Identified ectomycorrhizal fungi on the basis on the similarities with sequences given in the internet basis GenBank and UNITE and phylogenetic analyses

ECM type	Accession numbers of three best shot in GenBank and % of match and % of identity	Accession numbers of three best shot in UNITE and % of match and % of identity	Morphological-anatomical identifiication	Phylogenetical analysis
Unidentified type 1	*Meliniomyces* sp. KC007335.1 99% 99%; Uncultured *Hebeloma* JQ724056.1 99% 99%; Uncultured Helotiales DQ273322.1 99-98%	*Mollisia benesuada* | EstoniaUDB003038; *Crocicreas furvum* | Lithuania UDB003037; *Niptera dilutella* | Estonia UDB003005	/	/
Thelephora terrestris	*Thelephora terrestris* JQ711980.1 100-100%, HM189965.1 *Thelephora terrestris* 100-100%; *Thelephora terrestris* HQ406822.1 100-100%	Thelephoraceae | Slovenia UDB008264 100%; *Thelephora terrestris* | Estonia UDB003348 LOCKED by Urmas Kõljalg; *Thelephora terrestris* | Estonia UDB003346 99%	*Thelephora terrestris*	*Thelephora terrestris*
Tomentella ellisii	*Tomentella ellisii* DQ068971.1 100% 99%; Uncultured Thelephoraceae JN704829.1 100% 99%; Uncultured ectomycorrhiza (Tomentella) clone EU700261.1 97% 99%	*Tomentella ellisii* | Italy UDB016490 95%; *Tomentella ellisii* | Estonia UDB000219 96%; *Tomentella ellisii* | Finland UDB011603 LOCKED by Irja Saar	/	*Tomentella ellisii*
Unidentified type 2	Uncultured Pezizales clone P1_Contig_0290 JN704819.1 100% 99%; Uncultured ectomycorrhizal fungus clone Riv-5 EF484935.1 100% 99%; Uncultured ectomycorrhizal fungus clone unk1350 GU553372.1 100% 99%	*Sphaerosporella brunnea* | Finland UDB000994 94%; *Otidea alutacea* | Estonia UDB011428 98%; *Rhizina undulata* | Finland UDB016153 96%	/	/

TABLE 5. Average values (±standard error) of poplar roots colonization with ectomycorrhizal, arbuscular mycorrhizal and dark septated endophytic fungi at Timok site

Examined parameter	Average value
% Root length colonization with arbuscular mycorrhizal fungi	0
% Root length colonization with ectomycorrhizal fungi	18.19±3.47
% Root length colonization with endophytic fungi	2.23±0.50
% Root length colonization with other hyphae	1.80±565
Arbuscular mycorrhizal fungi/Ectomycorrhizal fungi	0

DISCUSSION

The main causes of extreme conditions at the pyrite tailings contaminated site near the river Timok were unfavourable water-air properties of analyzed soil, low pH and contamination with heavy metals copper and zinc. In such soils with high proportion of total sand, content of water available to the plants is low. Also, moderately acidic pH of the soil could not be favourable for while poplars that are known to prefer fluvisol soil type with slightly or moderately alkaline pH [32].

Analysis of fine roots' number, values of diversity indices and relative abundances of fungi that form ECM association with poplars from site Timok enabled comparison of ECM fungal community from studied site with ones from other similar sites.

On site Timok average number of fine roots per 1 dm^3 of soil was 12425.3/dm^3, while number of vital ECM roots was 2009.7/dm^3. Recorded values were much lower in comparison with results of Krpata *et al.* [33] who counted 1735-4263 mycorrhizal root tips in 100 ml of soil in aspen stand from site contaminated with heavy metals. On the other hand, in the experimental field with increased ozone concentration in the air, average value of fine poplar roots was 2599.1/dm^3 in control treated with water and 4573.5/dm^3 in the antiozonant protected plants [34].

Comparison of Shannon-Weaver diversity index recorded on site Timok (0.43) with its common values, which are in the range 1.5-3.5 according to Urbančič and Kutnar [35], showed that diversity on studied site was decreased. Low diversity of ECM types supports the observation that pollution could cause disappearance of sensitive ECM fungi and increase abundance of tolerant ones, decreasing in that way its diversity [36]. High proportion of sand in the soil and low content of water available to the plants (data not shown) suggested unfavourable conditions for development of mycorrhizal community. In addition, adverse influence of drought conditions on mycorrhizal fungi was proven by Lodge [37].

Under extreme abiotic conditions on site Timok only four ECM types were recorded. Similar results were obtained in studies of ECM communities on the sites under the influence of stress factors. On the site contaminated with heavy metals, Regvar *et al.* [38] recorded 7 ECM types on birch. On the clone *Populus nigra* × *maximowiczii cv.* Max grown as short rotation crop, Hrynkiewicz *et al.* [39] found 5 ECM types. On the other hand, Krpata *et al.* [33] recorded 54 ECM types on aspen from the site contaminated with heavy metals. On two sites polluted with zinc, Mleczko [40] recorded 23 ECM types, and the same number of ECM types was observed on uranium polluted site [42].

In our work data were collected in summer 2010 and 4 ECM types (*Thelephora terrestris*, *Tomentella ellisii* and two unidentified types) were recorded. On the same site in winter 2009, Katanić *et al.* [41] recorded 6 ECM types: *Tricholoma scalpturatum* (Fr.) Quel., *Tuber puberulum* Berk. & Br., *Thelephora* sp., Helotiales sp., Sebacinales sp. and Sordariomycetidae sp. Seasonal change in ECM community supports hypothesis of Koide *et al.* [43] that temporal distribution within ECM fungal community reduces competition among species.

Tomenteloid fungi belong to the most frequent and most abundant ECM partners of coniferous and broadleaved trees in woods of Europe and North America [44]. Kraigher and Al Sayegh Petkovšek [45] noted that, beside *Cenococcum geophilum,* fungus *Thelephora terrestris* is one of the rare fungi which form developed ECM in drought conditions. In addition, teleforoid fungi could play crucial role in forest ecosystems under the influence of stress [45]. Results from Timok are in accordance with their studies. Dominance of medium distance exploration type reveiled in our study is concordant with results of Rudawska *et al.* [46] who recorded high proportion of this exploration type on the locality contaminated with heavy metals.

High intraspecies variability could be found in ECM fungi according to Cairney [47], while Johnson *et al.* [48] consider that groups of genotypes can affect ecosystem processes in the same way as species do. Within species,

individuals differ a great deal in important reproductive and functional characters. Since, ECM fungus *Thelephora terrestris* is adapted to extreme conditions on the site Timok it could be assumed that strain from Timok developed some physiological adaptations to the extreme environmental factors.

In preparation of mycorrhizal inoculum for afforestration of demaged or contaminated sites, autochthonous strains of fungi, well adapted to such envionmental conditions, should be chosen [17, 49]. Strain of ECM fungus *Thelephora terrestris* from Timok could be proposed as a basis for creating inoculum for afforestation of this one or similar localities with extreme conditions.

CONCLUSIONS

According to the presented results it could be concluded that physico-chemical soil properties of studied site were unfavourable considering poor water properties, relatively low pH and high content of heavy metals (copper and zinc). Only four different ectomycorrhizal fungi were found from which fungus *Thelephora terrestris* made up 89% of all ectomycorrhizal roots. Total values of Species richness index and Shannon-Weaver diversity index were 0.80 and 0.43, respectively. No structures of arbuscular mycorrhizal fungi were recorded. The presented results suggest that described environmental conditions on investigated site caused decrease of ectomycorrhizal types diversity. Our findings point out that mycorrhyzal fungal community could be used as an appropriate indicator of environmental changes.

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