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Ectomycorrhizal fungal community associated with autochthonous white poplar from Serbia

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We analyzed the community of ectomycorrhizal fungi of an autochthonous white poplar (*Populus alba* L.) stand in the Kovilj-Petrovaradin marshes (Serbia), and examined its seasonal dynamics. Ectomycorrhizal types were identified by combining morphological and anatomical descriptions with molecular methods (sequencing of ITS region of ribosomal DNA). In two seasons, 20 ectomycorrhizal types were recorded, from which 11 types were identified to the species level, six were determined to the genus level, two types were determined to the family level and one type remained unidentified. Number of ectomycorrhizal types, number of fine roots, percentage of vital mycorrhizal roots, diversity indexes and abundance of exploration types did not differ significantly between autumn and spring. During both seasons, the most abundant types were: *Entoloma* sp., *Tuber maculatum*, *Cenococcum geophilum*, *Tuber rufum* and *Peziza* sp. Due to the high variation of the ectomycorrhizal types-based Shannon-Weaver diversity index in poplar stands, and the fact that poplars form dual mycorrhizal association, this index is not recommended as a reliable index for bioindication in poplar.

Keywords: Ectomycorrhiza, *Populus alba*, Diversity, Nature Reserve, Seasonal Dynamics, Morphological-Anatomical Characterization, Molecular Identification

Introduction

Poplars are world-wide distributed tree species that combine commercial importance with biotechnological advantages, such as rapid growth, simple *in vitro* propagation and availability of genetic transformation systems (Klopfenstein et al. 1997, Kaldorf et al. 2004). They are used in agroforestry systems (Eichhorn et al. 2006), short rotation forestry (Klašnja et al. 2006) and phytoremediation, as they cycle large amounts of water and grow rapidly by producing large amount of biomass, due to their deep root systems (Newman et al. 1997).

Poplars in plantations and natural stands regularly form dual associations with ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi (Karlinski et al. 2010) that are known to prefer different soil depths (Neville et al. 2002). While AM community has rarely attracted the interest of researchers, several prominent ECM species are thoroughly studied in poplars, in particular in white poplar (*Populus alba* L.), including the most expensive white truffle (*Tuber magnatum* Pico - Angelini & Granetti 1995).

Seedlings colonized with compatible ECM fungal species and strains are favored in making contacts with water and nutrients, as well as with other organisms in the soil (Kraigher 1996). In addition to the increased nutrient uptake, mycorrhizas offer numerous other benefits to symbiontic tree species, such as enhanced plant efficiency in absorbing water, reduced fertilization and irrigation requirements, increased drought tolerance, increased pathogen resistance, protection against damage from heavy metals and other pollutants, mitigation of various plant stresses, improvement of seedling growth and survival, and improvement of soil structure by the extramatrical hyphal network (Molina et al. 1992, Smith & Read 2008, Quoreshi 2008).

The functional compatibility and stress tolerance of ectomycorrhizal types is species specific and depend on both partners. Therefore, information on the ECM community structure can provide valuable information about physiology of forest trees and functioning of forest ecosystems (Kraigher et al. 2007).

Limited data on ECM community in white poplar from natural stands are available and its seasonal dynamics remain unclear (Jakucs 2002). The aim of this work was to investigate the ECM community in a case study location represented by an autochthonous white poplar stand in the Special Nature Reserve "Kovilj-Petrovaradin marshes" in Serbia and to analyze seasonal changes in the community between spring and autumn.

Materials and methods

Description of site and sampling

ECM roots were isolated from soil samples collected in the Special Nature Reserve, close to Novi Sad, Serbia (45° 12′ N, 19° 58′ E, elevation 78 m a.s.l.). According to the results from a nearby measuring station (Rimski Šančevi), the average annual precipitation (1951-2010) was 625 mm and the average yearly temperature was 11.4 °C (Stanojević 2012). Climate is a Dfa subtype of temperate continental climate with July and August as the warmest months (Republic Hydrometeorological Service of Ser-

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bia - http://www.hidmet.gov.rs/). The sampling site was occasionally flooded and the soil type was classified as fluvisol, loamy form (Katanić 2014). The sampling was performed in a naturally grown autochthonous white poplar (*Populus alba* L.) stand of 50-60 years old trees (100 trees ha^{-1}) mixed with scarcely abundant *Acer negundo* L. and *Robinia pseudoacacia* L.

Five mature white poplar trees were randomly selected. Two soil samples per tree were taken in autumn (September 2009) and spring (March 2010) at a distance of about 1 m from the tree trunk. In total, ten soil samples were collected in each season. A soil corer of 274 ml volume and reaching 18 cm depth was used for collecting standardized soil core samples (Kraigher 1999). Soil core samples were stored at 4 °C for up to one month. One day prior to analysis, soil samples were submerged in water and all fine roots were carefully washed from soil. Vital ECM root tips were separated from old, non-turgescent and non-mycorrhizal (ONN) root tips in water under a dissecting microscope. All fine root tip categories, including all identified types of ECM, were counted as total number in individual soil core sample.

Identification of ectomycorrhizae

ECM types were identified by combining morphological and anatomical approach with molecular methods. Morphological and anatomical characteristics of each ECM root type were assessed by a binocular Olympus SZX 12 (light source: Olympus Highlight 3100, daylight filter) and DIC (Nomarski) microscope Olympus BX 51 (magnification 100-2000×) following Agerer (1991), Kraigher (1996), and ECM descriptions published in Agerer (2008), Agerer et al. (2006), and Agerer & Rambold (2014). Based on the presence and abundance of emanating elements, ECM types were also classified into the exploration types proposed by Agerer (2001).

Molecular identification was based on nucleotide sequencing of ITS regions (Internal Transcribed Spacer) in nuclear ribosomal DNA. This is considered the best molecular marker for fungi identification (Kõljalg et al. 2013). After DNA extraction from 5-20 root tips with a PlantDNeasy® Mini Kit (Qiagen, Hilden, Germany) from each ECM type, the ITS region was amplified using the ITS 1f and ITS 4 primer pair (Gardes & Bruns 1993). 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 ECM fungi were determined by comparing with the sequences deposited in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/index.html) and Unite (Abarenkov et al. 2010) databases.

Data analysis

Diversity indexes were calculated per sample and per site (*i.e.*, by pooling the ECM community data) following the formulas given by Atlas & Bartha (1981) and Taylor et al. (2000):

(i) Species richness (d) = $(S - 1) / \log_{10} N$, where S is the number of ECM types and N is the number of all mycorrhizal tips;

(ii) Shannon-Weaver's diversity index (H) = $C/N\,(N \log N - \Sigma\, n_i \log n_i)$, where $C = 2.3$, N is the number of all mycorrhizal tips and n_i is the number of mycorrhizal tips of individual ECM type;

(iii) Evenness (e) = $H / \log S$, where H is the Shannon-Weaver's diversity index and S is the number of ECM types;

(iv) Equitability (J) = H/H_{max}, where H is the Shannon-Weaver's diversity index and H_{max} is the theoretical maximum H assuming that each ECM type was equally abundant;

(v) Berger-Parker's evenness index (BP) = 1 - (N_{max}/N), where N_{max} is the number of mycorrhizal tips of the most frequent ECM type and N is the number of all mycorrhizal tips.

Data of two soil core samples were joint and single tree was used as a statistical unit. The Student *t*-test was used to test the significance of differences in the number of ECM types, vital ECM root tips, old, non-turgescent and non-mycorrhizal roots, total fine roots, percentage of vital root tips and abundance of exploration types between autumn and spring. In order to fit the normal distribution, data were transformed as follows: count data were transformed according to square root transformation (Bartlett 1936), while percentage values were transformed according to arcsine transformation using the Bliss formula (Snedecor & Cochran 1976). The non-transformed data are presented in Tabs. 2 and 3. The Mann-Whitney U test was used to test the significance of differences in diversity indexes. All statistical analyses were performed using the package STATISTICA® version 12 (StatSoft Inc., Tulsa, OK, USA).

Tab. 1 - Identification of ectomycorrhizal (ECM) types from a mature white poplar (*Populus alba* L.) stand at the Special Nature Reserve Kovilj-Petrovaradin marshes (Serbia). Identifications are based on morphological-anatomical characters and nrITS DNA sequence comparison with the international nucleotide sequences databases. GenBank accession numbers are given for the analyzed ECM types. Exploration types were assessed after Agerer (2001).

Ectomycorrhizal type	Nucleotide database accession number	Exploration type
C. geophilum	HG937623	*short distance*
Genea verrucosa	HG937624	*short distance*
Hebeloma vaccinum	HG937625	*medium distance fringe subtype*
Hymenogaster tener	HG937626	*short distance*
Inocybe cf. meliolens	HG937629	*short distance*
Inocybe cf. rimosa	HG937627	*short distance*
Inocybe maculata	HG937628	*short distance*
Inocybe obsoleta	HG937630	*short distance*
Sebacina incrustans	HG937631	*short distance*
Tuber maculatum	HG937633	*short distance*
Tuber rufum	HG937632	*short distance*
Clavulina sp.	HG937634	*short distance*
Cortinariaceae sp.	HG937640	*short distance*
Entoloma sp.	HG937635	*medium distance smooth subtype*
Inocybe sp.	HG937636	*short distance*
Peziza sp.	HG937637	*contact*
Tomentella sp. 1	HG937638	*short distance*
Tomentella sp. 2	HG937639	*short distance*
Thelephoraceae sp.	HG937641	*short distance*
Unknown type KR	-	*short distance*

Results

Overall, twenty ECM types were determined at the examined site in the two investigated seasons. Eleven types were identified to the species level, six to the genus level, while other three were determined to the family level or remained unidentified (Unknown type KR - Tab. 1). *Cenococcum geophilum, Entoloma* sp., *Genea verrucosa, Inocybe maculata, Inocybe* sp., *Peziza* sp., *Sebacina incrustans, Tomentella* sp. 2, *Cortinariaceae* sp., *Tuber rufum* and *Tuber maculatum* were recorded in both seasons. *Hebeloma vaccinum, Inocybe* cf. *rimosa, Inocybe obsoleta, Thelephoraceae* sp., and *Tomentella* sp. 1 were observed only in spring, while *Clavulina* sp., *Hymenogaster tener, Inocybe* cf. *meliolens* and Unknown type KR were recorded only in autumn (Fig. 1a, Fig. 1b).

No significant differences between seasons were found as for the number of ECM types, vital ECM roots, old, non-turgescent and non-mycorrhizal roots, total number of fine roots and percentage of vital ECM root tips. However, all values were higher in spring (Tab. 2).

There were no significant differences between seasons in species richness index, Shannon-Weaver's index, Evenness, Equitability and Berger-Parker's index (Tab. 2). Diversity indexes revealed that number of species, relative abundance of individual

Fig. 1 - Ectomycorrhizal community structure in a mature white poplar stand in Serbia, based on sampling of 10 soil samples in spring (a) and 10 soil samples in autumn (b).

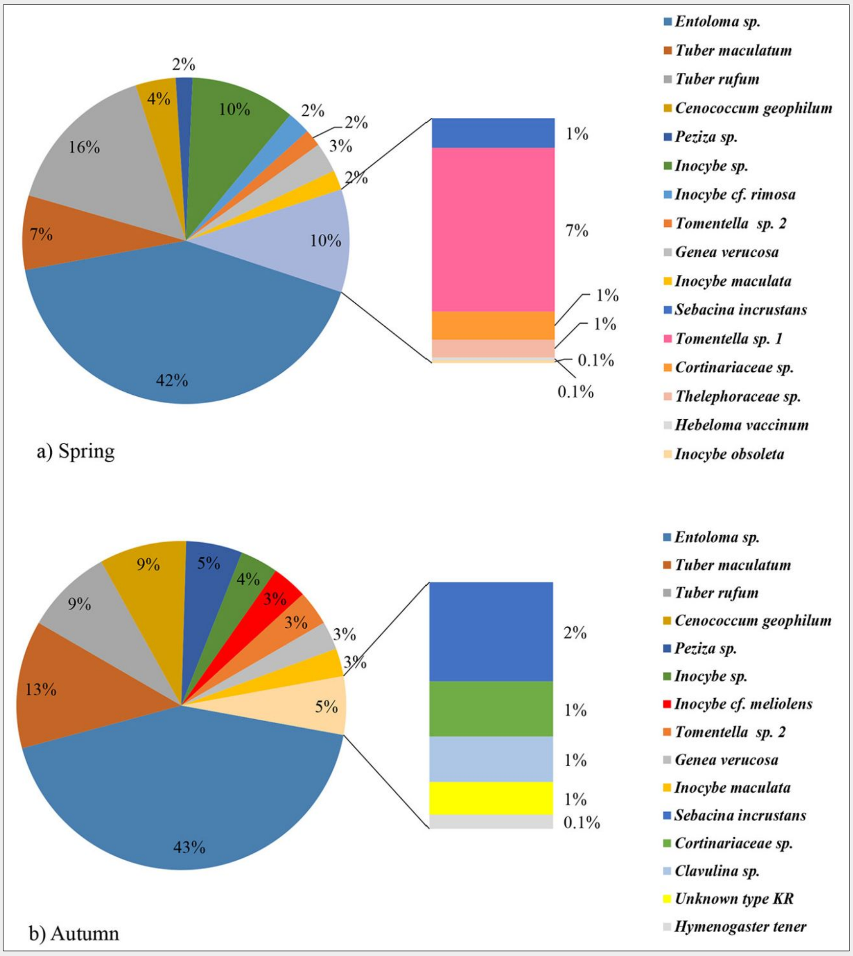
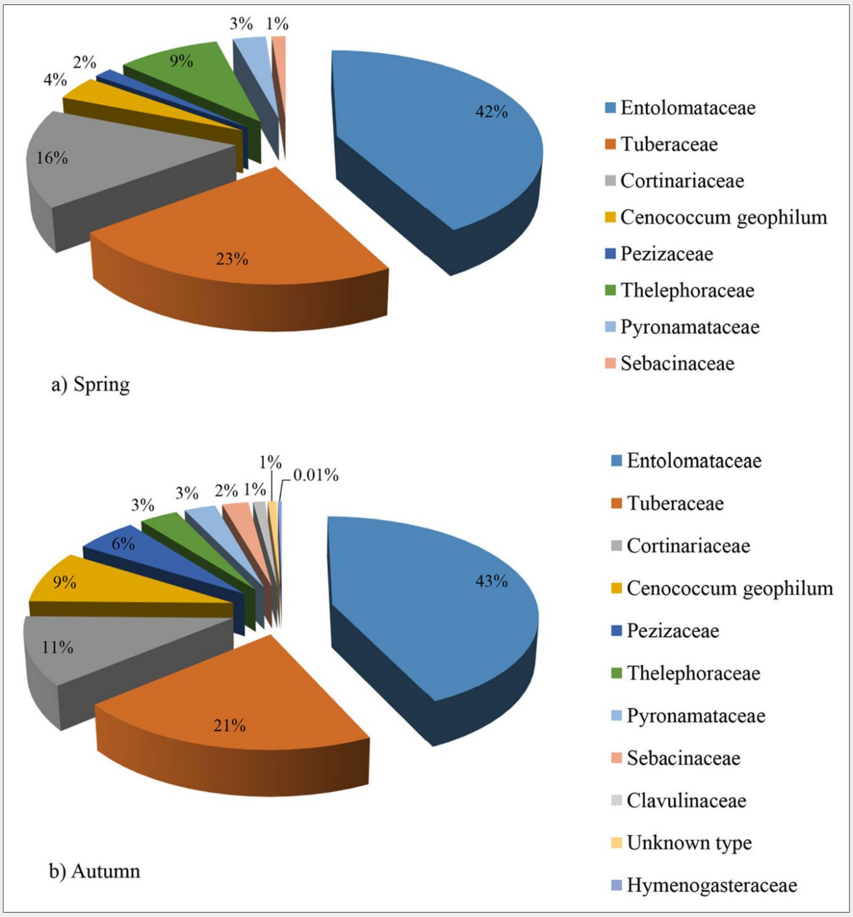


Fig. 2 - Relative abundance of ectomycorrhizal fungal families in a mature white poplar stand in Serbia, based on sampling of 10 soil samples in spring (a) and 10 soil samples in autumn (b)



Tab. 2 - Comparison of total and average values (± standard erorr) of number of ectomycorrhizal (ECM) types, vital ECM root tips, old, non-turgescent and non-mycorrhizal roots (ONN), total fine roots and diversity indexes from a mature white stand located in Serbia between spring and autumn. (a): p-values after the *t*-test; (b): p-values after the Mann-Whitney's U test.

Parameter	Spring		Autumn		p-value
	Total value per site	Average value per tree	Total value per site	Average value per tree	
Number of ECM types	16	5.10 ± 0.60	15	4.90 ± 0.20	0.838[a]
Number of vital ECM root tips	4300	430 ± 133.6	3030	303 ± 65.5	0.511[a]
Number of ONN root tips	22140	2214 ± 511.6	19335	1933.5 ± 148.2	0.686[a]
Total number of fine root tips	26440	2644 ± 607.7	22365	2236.5 ± 141.9	0.609[a]
% of vital ECM Root tips	16	16.20 ± 2.90	14	13.90 ± 3.10	0.569[a]
Species richness index	4.13	1.62 ± 0.20	4.02	1.63 ± 0.08	0.754[b]
Shannon-Weaver index	1.95	1.01 ± 0.12	2.00	1.15 ± 0.06	0.403[b]
Evenness	0.70	0.626 ± 0.06	0.74	0.723 ± 0.03	0.174[b]
Equitability	1.62	1.44 ± 0.13	1.70	1.67 ± 0.07	0.174[b]
Berger-Parker index	0.58	0.38 ± 0.04	0.57	0.48 ± 0.04	0.117[b]

species, equitability and evenness between species, and dominance rate of the most abundant species did not differ significantly between seasons, *i.e.*, the ECM community structure did not change across seasons.

In both seasons, the same five ECM types dominated and represented about 80% of the total number of ECM root tips. The most abundant ECM type was *Entoloma* sp. with over 40%, followed by *Tuber maculatum, Cenococcum geophilum, Tuber rufum* and *Peziza* sp. Relative abundance of these ECM types was similar in both seasons (Fig. 1a, Fig. 1b).

The most abundant ECM fungal family was Entolomataceae, followed by Tuberaceae and Cortinariaceae. In both seasons the three above-mentioned families represented 70% of the total number of all ECM root tips (Fig. 2a, Fig. 2b).

In terms of species richness, the family Cortinariaceae was the richest, with six species in spring and four in autumn (Fig. 3). The number of Basidiomycota ECM symbionts was higher than those of Ascomycota, both in spring (11 *vs.* 5, respectively) and in autumn (10 *vs.* 5, respectively). Similarly, the contribution of root tips colonized by Basidiomycota ECM fungi was higher than those colonized by Ascomycota fungi (in spring 68.5 *vs.* 31.5 %, respectively, in autumn 62 *vs.* 38%, respectively - data not shown).

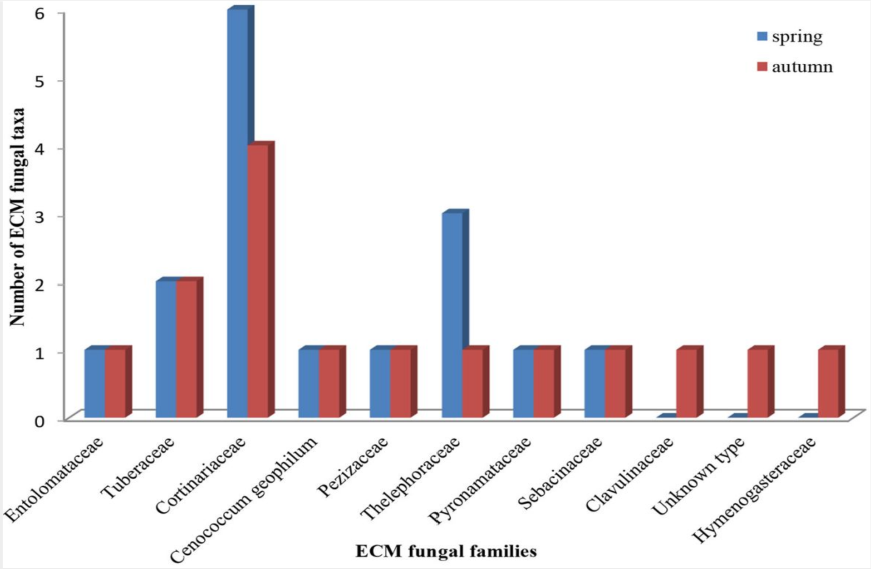


Fig. 3 - Number of ectomycorrhizal fungal taxa within fungal families recorded in mature white poplar stand located in Serbia in spring and autumn.

Tab. 3 - Relative abundance (± standard error, %) of ectomycorrhiza exploration types in a mature white poplar stand in Serbia in spring and autumn, and significance of Student *t*-test for the effect of season.

Exploration type	Spring	Autumn	*t*-test (p-value)
Contact type	1.87 ± 1.01	2.59 ± 2.59	0.754
Short distance	65.23 ± 9.38	60.68 ± 8.42	0.648
Medium distance	32.90 ± 9.09	36.72 ± 9.09	0.724
Long distance	0	0	-

The most abundant exploration type (ET) was short-distance ET followed by medium -distance ET and contact ET. Long-distance ET was not observed. Abundance of each ET did not significantly differ between the seasons (Tab. 3).

Discussion

The community of ectomycorrhizal fungi in this case study represented by a mature autochthonous white poplar stand located in the Special Nature Reserve "Kovilj-Petrovaradin marshes" (Serbia) is one of the few studies (Jakucs 2002, Katanić et al. 2010, Katanić 2014) on ECM diversity in white poplar and, up to our knowledge, the only one in a mature natural stand.

The average amount of vital ECM root tips (1106 dm^{-3} in autumn and 1570 dm^{-3} in spring) was considerably lower than in mature stands of other species such as beech (1460-15 800 dm^{-3} - Mašek & Grebenc 2011) and spruce (4309-6716 dm^{-3} - Kraigher 1999). The number of all fine roots per soil volume in poplar stands was even more variable. In this study, such value was 9651 dm^{-3} in spring and 8163 dm^{-3} in autumn, while ranged from 2599 dm^{-3} in control plants irrigated with water to 4573 dm^{-3} in plants treated with the anti-ozonant ethylenediurea in an ozone-sensitive clone (Katanić et al. 2014). Krpata et al. (2008) counted 17 350-42 630 dm^{-3} ECM root tips at a site with aspen contaminated by heavy metals. The relatively low number of ECM roots in our white poplar stand reflects the regular flooding occurring in the studied area and the dual colonization with ECM and AM fungi. In the same poplar trees, Katanić et al. (2013) recorded values of root length colonization with ECM and AM fungi of 16.7 and 8.8 %, respectively.

The 20 ECM types recorded in total are a considerably lower number compared to some previous studies. On individual *Populus tremula* trees in an old-growth mixed forest, Bahram et al. (2011) found 122 ECM fungal species. Analyzing ECM community at two sites with 30-40 m tall white poplars during three years, Jakucs (2002) recorded 70 ECM types, while Krpata et al. (2008) found 54 ECM types at a heavy metal polluted site with 20 to 25-year-old aspen trees. However, results of our research are in accordance with Visser et al. (1998), who recorded 22 ECM types in a mixed forest dominated by American aspen, and Kaldorf et al. (2004), who observed 23 morphotypes in a five-year-old experimental aspen plantation. In addition, at three experimental sites with poplars Karlinski et al. (2013) found in total 27 ECM fungal taxa. In a white poplar plantation, Katanić et al. (2010) preliminary recorded 15 ECM types. However, when ECM diversity was studied seasonally, a total of 30 ECM types were observed (Katanić 2014). It seems that the number of ECM fine roots and the diversity of ECM types in poplar are highly variable, and the naturalness of the studied Special Nature Reserve does not contribute to increase ECM diversity as compared with other poplars' sites.

Values of Shannon-Weaver's diversity index (1.95 in spring and 2.00 in autumn) are in accordance with results obtained by DeBellis et al. (2006). These authors recorded a similar Shannon-Weaver diversity index of 2.00 in an aspen-dominated plot by using morphological characterization of ECM types, but a considerably higher index was recorded (3.00) when molecular identification tools were applied. Values of the Shannon-Weaver's diversity index obtained in extreme conditions are controversial. On the one hand, an ozone sensitive poplar clone showed lower values of Shannon-Weaver's index, namely 1.60 in anti-ozonant protected plants and 1.21 in control plants (Katanić et al. 2014). On the other hand, Krpata et al. (2008) recorded relatively high value of Shannon-Weaver's diversity index (2.00) in aspen trees grown at a site contaminated with heavy metals. Due to the high variation of the ECM-based Shannon-Weaver's diversity index for poplar stands, and the fact that poplars form dual mycorrhizal association, this index can not be considered reliable for bioindication, as otherwise proven for spruce (Kraigher 1999). None of the above-mentioned authors compared fine roots data, species abundance or diversity indexes of poplar stands among seasons. However, in our study differences between seasons did not show any significant effect on diversity of the ECM community.

ECM community associated with white poplars in the Kovilj-Petrovaradin marshes consisted of few abundant and numerous infrequent ECM types, in accordance with previous research (De Roman & De Miguel 2005, O'Hanlon & Harrington 2012). Koide et al. (2007) reported abundant ECM types present in both seasons with similar relative abundance, while some rare species were specific for autumn or spring only. In both seasons, five ECM types made up near 80% of all ECM root tips, while 11 or 10 ECM types (in spring and autumn, respectively) contributed to the rest. This finding is in accordance with the evidence that the ECM community varied according to a logarithmic distribution of abundances (Dahlberg 2001, Pickles et al. 2010).

In the Kovilj-Petrovaradin marshes, the genus *Inocybe* was the most abundant one, with five ECM types, while genera *Tuber* and *Tomentella* had two members. Analyzing the diversity of mycorrhizal fungi in mixed deciduous stand, Lang et al. (2011) noticed that *Tomentella* and *Inocybe* were the most abundant and contributed mostly to the species richness. In the ECM community associated with aspen grown at the site contaminated with heavy metals, the most abundant taxonomic groups were *Tomentella* (17), *Inocybe* (6), *Cortinarius* (5), *Hebeloma* and *Tuber* with 3 operational taxonomic units (Krpata et al. 2008). At three sites with poplar clones, Karlinski et al. (2013) recorded the dominance of Cortinariaceae, Thelephoraceae and Tricholomataceae that constituted nearly 90% of the mycorrhizal community.

Inocybe species are well-known colonizers of ECM plants on disturbed or pioneer sites (Nara et al. 2003). Investigating mycorrhizal fungi associated with aspen at three different sites, Cripps (2003) observed 54 ECM fungi and 14 species belonged to the genus *Inocybe*. Tomenteloid fungi are the most frequent and widespread ECM partners of deciduous and evergreen tree species in the forests of Europe and North America (Gardes & Bruns 1996, Dahlberg 2001). In the white poplar forest adapted to dry conditions of the Hungarian plain, *Tomentella* group was a minority component of the ECM community (Jakucs 2002). Similar observations were made in the occasionally flooded Kovilj-Petrovaradin marshes. Investigating the molecular diversity and the ecological specificity of truffles originating from the mid-west of Balkan Peninsula, Marjanović et al. (2010) recorded 12 species from this group, including *Tuber rufum* and *T. maculatum. Cenococcum geophilum* is a cosmopolitan ECM fungus with a wide range of hosts and habitats. It is a frequent and abundant ECM type adapted to environment under stress (LoBuglio 1999) that was recorded in almost half of the samples in our study. Di Pietro et al. (2007) noted that under extreme stress, ECM formed by *Cenococcum geophilum* has the ability to survive better than ECM of some other fungi, and that this species is particulary efficient in the protection of fine roots from drought stress. However, during the three-year survey of two ECM communities associated with poplars in dry conditions, Jakucs (2002) did not observe the presence of *C. geophilum*, while in our study it was found in an area that is occasionally flooded.

Although the differences were not statistically significant, all parameters investigated showed slightly higher values in spring than in autumn. This is in accordance with Courty et al. (2008) who recorded the highest number of vital ECM roots in April, while the lowest value was observed in September. The absence of significant differences between autumn and spring in the number of ECM types and diversity indexes in this study is in accordance with the results of De Roman & De Miguel (2005). In both seasons the same three fungal families were the most abundant (Entolomataceae, Tuberaceae and Cortinariaceae). In contrast, Richard et al. (2011) found that the relative abundance of two out of three the most abundant families (Russulaceae and Cortinariaceae) showed significant seasonal shifts.

In both seasons, Basidiomycota dominated the examined ECM community, confirming numerous studies on poplars (Cripps 2001, 2003, Krpata et al. 2008, Karlinski et al. 2013, Katanić et al. 2014) where the Basidiomycota group was the most abundant and had more members in comparison to Ascomycota.

The most abundant exploration type in both seasons was short-distance ET and then medium-distance ET. Agerer (2001) found a relationship between ETs and their potential ecological roles. Jakucs (2002) noticed that in two forests adapted to similar ecological conditions, dominant ECM types belonged to the same ET. Analyzing ET of ECM community, Rudawska et al. (2011) concluded that abundance of a particular ET is related to the soil chemistry, since occurrence of contact ET was related to high nutrient content. On the other hand, medium-distance fringe exploration type was abundant at a sites contaminated with heavy metals (Rudawska et al. 2011, Karlinski et al. 2013). We assume that the similar distribution of ETs observed at our site in spring and autumn results from similar ecological conditions.

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