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Eutypella parasitica and Other Frequently Isolated Fungi in Wood of Dead Branches of Young Sycamore Maple (*Acer pseudoplatanus*) in Slovenia

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Abstract: *Eutypella parasitica* R.W. Davidson and R.C. Lorenz is the causative agent of Eutypella canker of maple, a destructive disease of maples in Europe and North America. The fungus *E. parasitica* infects the trunk through a branch stub or bark wound. Because the fungal community may have an impact on infection and colonization by *E. parasitica*, the composition of fungi colonizing wood of dead branches of sycamore maple (*Acer pseudoplatanus* L.) was investigated in five sampling sites in Slovenia. Forty samples from each sampling site were collected between the November 2017 and March 2018 period. Isolations were made from the wood in the outer part of dead branches and from discoloured wood in the trunk that originated from a dead branch. Pure cultures were divided into morphotypes, and one representative culture per morphotype was selected for further molecular identification. From a total of 2700 cultured subsamples, 1744 fungal cultures were obtained, which were grouped into 212 morphotypes. The investigated samples were colonized by a broad spectrum of fungi. The most frequently isolated species were *Eutypa maura* (Fr.) Sacc., *Eutypa* sp. Tul. and C. Tul., *Fusarium avenaceum* (Fr.) Sacc., *Neocucurbitaria acerina* Wanas., Camporesi, E.B.G. Jones and K.D. Hyde and *E. parasitica*. In this study, we distinguished species diversity and the fungal community. There were no significant differences in the diversity of fungal species between the five sampling sites, and branch thickness did not prove to be a statistically significant factor in fungal species diversity. Nevertheless, relatively low Jaccard similarity index values suggested possible differences in the fungal communities from different sampling sites. This was confirmed by an analysis of similarities, which showed that the isolated fungal community distinctly differed between the five sampling sites and between the different isolation sources. *Eutypella parasitica* was isolated from all five investigated sampling sites, although Eutypella cankers were observed in only three sampling sites, indicating the possibility of asymptomatic infection.

Keywords: fungal composition; invasive species; diversity; fungal communities; molecular identification; frequencies; Jaccard similarity index; *Eutypella parasitica*; analysis of similarities; colonization

1. Introduction

Sycamore maple (*Acer pseudoplatanus* L.) is the most common maple and also one of the most valuable tree species in Europe [1]. It is a temperate climate tree originating from the mountainous areas of Central Europe. The current distribution of *A. pseudoplatanus* extends from Turkey and Spain to Ireland and Sweden [2], and it is adapted to a wide range of site conditions [3]. It is characterized by rapid growth and potentially high timber prices [4]. It is light demanding and grows best on highly

productive sites [5–7]. In 2018, *A. pseudoplatanus* represented 3.14% of the wood stock of Slovenian forests [8].

Nectria cinnabarina (Tode) Fr., *Cryptostroma corticale* (Ellis and Everh.) P.H. Greg. and S. Waller, *Rhytisma acerinum* (Pers.) Fr., *Verticillium dahliae* Kleb., *Cristulariella depraedans* (Cooke) Höhn., *Sawadaea* Miyabe, *Diplodina acerina* (Pass.) B. Sutton, *Acericecis vitrina* Kieffer, *Zeuzera pyrina* L. and *Eriophyes* Nalepa are the most typical harmful organisms for *A. pseudoplatanus* worldwide [9]. The fungus *Eutypella parasitica* R.W. Davidson and R.C. Lorenz, the causative agent of Eutypella canker of maple, has also a high potential to damage sycamore maple. The disease was reported for the first time in Europe from Slovenia [10], and later from Austria, Croatia, Germany, Hungary, the Czech Republic, Poland and Italy [11–17]. It is a serious disease that affects the aesthetic and economic value of infected maple trees [10]. It is believed to originate from North America [18] and represents a considerable risk for an extensive area of naturally distributed maples in Europe [17,19]. *Eutypella parasitica* is believed to enter the trunk through branch stubs or bark wounds [20] and consequently creates a characteristic canker mostly on the lower portions of the trunk [18,21]. Fruiting bodies (i.e., perithecia) develop in the central part of six to eight-year-old cankers. Their black necks protrude slightly above the surface [18] and release ascospores during wet periods at moderate temperatures [20,22]. The high number of discharged ascospores is an important factor of successful disease spread [21]. Spores are dispersed by wind, over long distances by trade of plants for planting or wood [23]. The optimal temperature for fungus growth is 24–28 °C [22,24].

Fungal communities in the dead branches of *A. pseudoplatanus* and other maples are still not well known. There have been very few studies of the fungal endophytes and saprotrophs present on sycamore branches, and none of these species have been studied in connection with *E. parasitica*. Most fungi on the dead twigs of *A. pseudoplatanus* belong to Ascomycota and Deuteromycota [25]. Fungal communities of Aceraceae are usually dominated by a few species that belong to the Diaporthales [26]. Different authors [25,27–31] have studied the fungal communities of the wood and bark of living or dead branches of *A. pseudoplatanus* (Table 1). These studies provide a context for this study and a reference point for comparison with our results. There are also some research papers on studies of fungal communities in *Acer saccharum* Marshall [32], *A. ginnala* Maxim. [33], *A. truncatum* Bunge [34] and *A. rubrum* L. [35], but extensive research of fungi in wood of dead branches of *A. pseudoplatanus* in connection with *E. parasitica* is lacking.

The aim of our study was to determine the species composition of fungi colonizing wood of dead branches of young *A. pseudoplatanus* in connection with *E. parasitica* in the central part of Slovenia.

Table 1. Prevailing fungal taxa [6] isolated from the branches of *Acer pseudoplatanus* in other studies.

	Isolation Source	Fungal Taxon
Butin and Kowalski [25] [1]	dead twigs	*Aposphaeria* sp. Berk., *Diaporthe acerina* (Peck) Sacc., *Durella atrocyanea* (Fr.) Höhn., *Durella commutata* Fuckel, ***Eutypa maura* (Fr.) Sacc.**, *Fusarium stilboides* Wollenw., *Pezicula acericola* (Peck) Peck ex Sacc. and Berl., ***Phialocephala* sp. W.B. Kendr.**, ***Phomopsis pustulata* (Sacc.) Died.**, *Prosthecium platanoidis* (Pers.) M.E. Barr, ***Splanchnonema pupula* (Fr.) Kuntze**
Chlebicki [27] [2]	wood of dead branches	***Eutypa maura* (Fr.) Sacc.**, ***Nectria cinnabarina* (Tode) Fr.**, *Prosthecium platanoidis* (Pers.) M.E. Barr, *Prosthecium pyriforme* Jaklitsch and Voglmayr
Kowalski and Kehr [28] [3]	living branch bases	*Aposphaeria* sp. Berk., *Diplodina acerina* (Pass.) B. Sutton, *Mollisia* sp. (Fr.) P. Karst., ***Petrakia irregularis* Aa**, *Pezicula cinnamomea* (DC.) Sacc., *Phialocephala dimorphospora* W.B. Kendr., ***Phomopsis* sp. Sacc. and Roum.**, ***Phomopsis pustulata* (Sacc.) Died.**, ***Splanchnonema pupula* (Fr.) Kuntze**, *Torula* sp. Pers.
Ellis and Ellis [29] [4]	wood and bark	***Eutypa maura* (Fr.) Sacc.**, ***Phomopsis pustulata* (Sacc.) Died.**, ***Splanchnonema pupula* (Fr.) Kuntze**, ***Xylaria longipes* Nitschke**
Unterseher, Otto and Morawetz [30] [5]	dead canopy twigs	*Auricularia auricula-judae* (Bull.) Quél., ***Eutypa maura* (Fr.) Sacc.**, ***Nectria cinnabarina* (Tode) Fr.**, *Peniophora cinerea* (Pers.) Cooke, *Peniophora lycii* (Pers.) Höhn. and Litsch., *Prosthecium acerinum* Voglmayr and Jaklitsch, *Prosthecium pyriforme* Jaklitsch and Voglmayr, ***Schizophyllum commune* Fr.**, *Trichoderma viride* Pers.,
Johnova [31] [4]	decayed trunks, stumps, twigs and branches	***Trichoderma spp.* Pers.** ***Eutypa lata* (Pers.) Tul. and C. Tul.**, ***Neonectria coccinea* (Pers.) Rossman and Samuels**, ***Xylaria longipes* Nitschke**

[1] Frequency > 6%; [2] most common species; [3] frequency > 10%; [4] only species also found in our study; [5] frequency > five samples; [6] species found in our study are bolded.

2. Materials and Methods

2.1. Definitions of Repeatedly Used Terms

We use a number of terms repeatedly throughout the text. Short explanations of these terms are given here for easier reading and understanding:

- Sampling site—a site or an area in the forest stand where samples were collected
- Sample—a dead *A. pseudoplatanus* branch with a section of the trunk where it was attached (Figure 1)
- Isolation source—a location in a sample from which subsamples were cut (B—branch; T—trunk; C—control) (Figure 1)
- Subsample—a small piece of approximately 1 × 2 × 2 mm cut from the wood and representing three isolation sources (if possible) in each sample
- Culture—an outgrown mycelium from a subsample

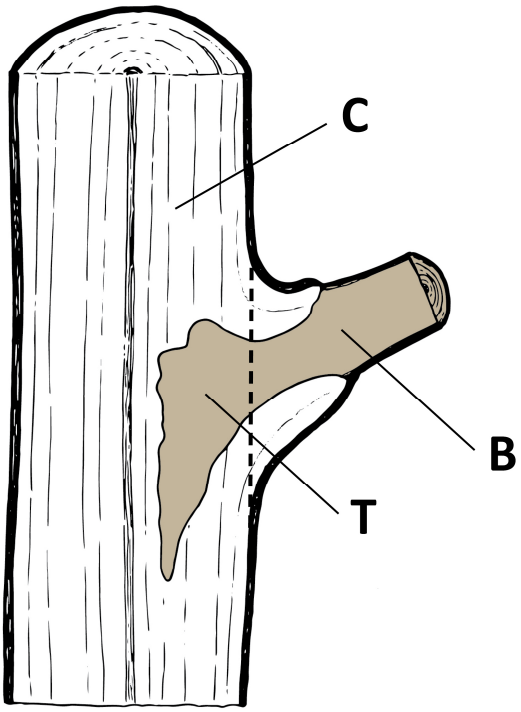

Figure 1. Sources of fungal isolations in a sample: B, branch; T, trunk; C, control (illustration by S. Zidar, Slovenian Forestry Institute).

2.2. Inventory of Eutypella Canker of Maple

One-hectare plots (100 × 100 m) were established in five sampling sites (Table 2) to assess the presence of the Eutypella canker of maple. Every sycamore maple with a diameter at breast height of at least ten centimetres was carefully checked for typical symptoms of *E. parasitica*—depressed or flattened areas covered by firmly attached bark; broad, slightly raised concentric rings of callus tissue; white to buff mycelial fans under the bark at the margins of the canker; and the presence of black perithecia in the centres of older cankers [18].

Table 2. Characteristics of sampling sites.

Sampling Site	Label	Longitude (°E)	Latitude (°N)	Elevation (m a.s.l.)	Relief Aspect	T (°C) [1]	P (mm) [2]
Rožnik	R	14.48723	46.06508	345	NE	10.0	1424
Smrekovec	SM	14.50187	45.88679	835	SW	7.7	1652
Mokrc	M	14.50965	45.88135	860	SW	6.8	1702
Mala voda	MV	14.26736	46.04212	536	NW	9.1	1657
Samotorica	S	14.25378	46.03027	647	S	9.1	1636

[1] Mean annual temperature (°C) average from 1981 to 2010 [36]. [2] Annual precipitation sum (mm) average from 1981 to 2010 [37].

2.3. Sampling

Field sampling was performed between the November 2017 and March 2018 period. In each sampling site, twenty individuals of *A. pseudoplatanus* with a diameter at breast height of less than 6.5 cm were randomly chosen. From each tree, one to three samples were randomly collected (Figure 1) and altogether 40 samples were collected from each sampling site. A total of 200 samples were analysed (Table 3).

Table 3. Mean and standard deviation (in parenthesis) of the height, length and DBH (diameter at breast height) of sampled trees and branches in sampling sites.

Sampling Site	DBH (cm)	Tree Height (m)	Branch Height [1] (m)	Branch Length (cm)	Branch Diameter at Branch Base (cm)	Trunk Diameter at Branch Base (cm)
Rožnik	3.7 (1.0)	5.42 (1.69)	2.19 (1.29)	28.6 (26.9)	1.0 (0.5)	3.7 (0.8)
Smrekovec	2.8 (0.6)	4.64 (0.51)	1.25 (0.61)	63.2 (58.7)	0.8 (0.3)	3.2 (0.7)
Mokrc	2.7 (0.6)	4.28 (0.89)	1.18 (0.79)	28.1 (25.1)	0.6 (0.3)	3.1 (0.7)
Mala voda	4.0 (0.7)	6.96 (1.66)	2.49 (1.39)	10.0 (0.0)	1.5 (0.7)	3.8 (1.1)
Samotorica	4.3 (0.9)	8.49 (2.05)	3.27 (1.31)	5.4 (2.6)	1.5 (0.5)	3.8 (1.1)

[1] Height on the trunk where the branch was attached to the trunk.

2.4. Isolation of Fungi

Collected samples were labelled, placed in paper bags and transported to the laboratory. Samples were stored at 4 °C and processed within two days. After rinsing and scrubbing under running tap water, samples were surface sterilized by 70% (v/v) ethanol (1 min), followed by sodium hypochlorite with 1% available chlorine (30 sec) and again by 70% (v/v) ethanol (1 min). Finally, samples were rinsed under distilled water. After surface sterilization, samples were dried, halved and cut into smaller subsamples with sterilized equipment. Fungal isolations were made from wood representing three sources (if possible) in each sample: wood in the outer part of the dead branch (eight subsamples; labelled "B"; branch), discoloured wood in the trunk that originated from the dead branch (eight subsamples; labelled "T"; trunk) and visually healthy, non-discoloured wood in the trunk (four subsamples; labelled "C"; control) (Figure 1). Subsamples were evenly plated on 2% (w/v) malt extract agar (MEA; Becton Dickinson, Sparks, MD, USA), four subsamples per plate (70 mm in diameter). Petri dishes were sealed, incubated at 19.6 °C ± 1.0 °C and examined periodically. Outgrown mycelium from the wood subsamples were immediately transferred to new Petri dishes with 2% (w/v) MEA.

Obtained fungal cultures were grouped into morphotypes according to the morphological characteristics of the mycelium cultures. One representative culture from those morphotypes, with more than five cultures, was selected for further molecular identification. Representative cultures were deposited in the culture collection of the Laboratory of Forest Protection at the Slovenian Forestry Institute.

2.5. DNA Extraction, Amplification and Sequencing

Genomic DNA was extracted from the mycelium scraped from the MEA plates using a NucleoSpin® Plant II (Macherey Nagel, Düren, Germany) following the manufacturer's instructions, after homogenizing the fungal material with a Lysing Matrix A tube (MP Biomedicals, Solon, OH, USA) using a Precellys Evolution device (Bertin Technologies, Montigny-le-Bretonneux, France). The ITS rDNA region was amplified using primer pairs ITS1 and ITS4 [38]. The 50 µL PCR mixture consisted of PCR® Master Mix (2x) (Thermo Fisher Scientific, Waltham, MA, USA), 1 µL each of 10 µM primers (Sigma-Aldrich, St. Louis, MO, USA), 3 µL of DNA (approx. conc. 25 µg/mL) and 20 µL of molecular grade water (Sigma-Aldrich, St. Louis, MO, USA). The reaction conditions were as follows: 3 min initial denaturation at 95 °C, followed by 39 cycles of 30 s denaturation at 95 °C, 45 s primer annealing at 55 °C and 90 s extension at 72 °C, and a final extension at 72 °C for 10 min.

For determination of *Fusarium* spp., nucleotide sequences of elongation factor (EF-1α) were amplified using primers EF1 and EF2 [39]. The reaction conditions were as follows: 5 min initial denaturation at 95 °C, followed by 45 cycles of 30 s denaturation at 95 °C, 30 s primer annealing at 51.5 °C and 60 s extension at 72 °C, and a final extension at 72 °C for 6 min.

The obtained PCR products were cleaned using a Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI, USA) kit according to the manufacturer's protocol and sequenced at the DNA sequencing facility of Eurofins Genomics (Ebersberg, Germany) in both directions. Sequences were visualised and manually edited using Geneious Prime® version 2019.0.4 (Biomatters Ltd.,

Auckland, New Zealand). Each consensus sequence, representing one morphotype, was used to perform individual searches with the BLASTn algorithm against nr/nt database from the NCBI website on different dates from 23 January to 2 August 2019. Sequences were deposited in GenBank.

2.6. Data Analysis

The colonization rate was calculated as the total number of infected subsamples (subsamples with outgrown mycelium) divided by the total number of incubated subsamples [40]. The relative colonization frequency (*F*) of an individual taxon was expressed as the number of cultures of a certain species divided by the total number of cultures. The density index (*DI*) was defined as the number of cultures produced by one species divided by the number of samples in which the species was present [28].

The Shannon diversity index (*H′*) of fungal taxa was calculated from the equation:

$$H' = -\sum p_i \ln(p_i) \quad (1)$$

where p_i is the proportion of individuals found in the *i*th species [41], to measure the diversity of populations in different sampling sites and isolation sources. To obtain more information from the Shannon diversity index, we calculated the corresponding effective number of species [42] for each sampling site and isolation source:

$$ENS = \exp(H') \quad (2)$$

The species evenness (*J′*) was estimated according to Pielou's formula:

$$J' = H'/H_{max} \quad (3)$$

where *H′* represents the Shannon diversity index, and $H_{max} = \ln(S)$ (*S* is the species richness, defined as the number of species of a given taxon) [41]. The Jaccard similarity index (C_J) [41] was used to compare fungal communities from different sampling sites:

$$C_J = a/(a + b + c) \quad (4)$$

where *a* is the total number of species present in both sampling sites, *b* is the number of species present only in site 1 and *c* is the number of species present only in site 2.

To study frequencies and diversity of sample colonization by the most common fungal taxa in relation to branch base diameter, we designed three branch thickness classes: 0.2–0.7 cm, 0.8–1.2 cm and 1.3–3.2 cm. They covered approximately the same number of branches in individual classes and had more or less equal diameter.

Calculations of colonization rate, relative colonization frequency, density index and the Jaccard similarity index were performed in Microsoft Excel version 1902, while the Shannon diversity index, effective number of species, species evenness, t-tests and analysis of similarities were performed in the R software environment for statistical computing [43] with the "vegan" library [44]. The Mann–Whitney U test was used to compare the diversity index between samples with and without *E. parasitica*. Similarly, the Kruskal–Wallis test was used to compare the diversity index between different sampling sites, between different isolation sources and between different branch base diameter classes. Differences in fungal community structure between sampling sites, isolation sources, branch base diameter classes and samples with or without *E. parasitica* were tested with an analysis of similarities (ANOSIM) based on Jaccard dissimilarities. A non-parametric test, ANOSIM uses ranked dissimilarities instead of raw data and is similar to an ANOVA hypothesis test. ANOSIM is used to determine if the differences between two or more groups are significant [45].

3. Results

3.1. Eutypella Parasitica

The Eutypella canker of maple was observed in three sampling sites—Rožnik, Mala voda and Smrekovec (Table 4). The highest number of symptomatic sycamore maples were observed at Rožnik and Smrekovec (1.39% and 1.29% of surveyed maples). In contrast, only one of 216 surveyed maples from Mala voda exhibited a typical canker, and the field survey did not detect the Eutypella canker of maple in Mokrc and Samotorica.

Table 4. Eutypella canker of maple: number of visually healthy and symptomatic *A. pseudoplatanus* in sampling sites.

Sampling Site	Visually Healthy	Symptomatic
Rožnik	213	3
Smrekovec	153	2
Mokrc	92	0
Mala voda	215	1
Samotorica	314	0

Eutypella parasitica was isolated in all sampling sites. It was most frequently found in Rožnik (61.3%), but in other sampling sites, the relative colonization frequency was much lower (3.2–14.5%) (Table 5). The fungus represented 8.5% of all isolations from Rožnik, 2.6% from Mokrc, 1.4% from Samotorica, 0.9% from Smrekovec and 0.5% from Mala voda. The species yielded 62 colonies from 19 different samples. With a density index of 3.26, it was one of the most densely occurring species (Table 6). *Eutypella parasitica* was 1.5 times more frequent in the discoloured wood of trunks (T) than in the outer parts of dead branches (B).

Table 5. Relative colonization frequencies (*F*) of subsamples by *E. parasitica*.

Sampling Site	Rožnik		Smrekovec		Mokrc		Mala voda		Samotorica	
Isolation Source	T [1]	B [2]	T	B	T	B	T	B	T	B
F (%)	46.77	14.52	3.23	3.23	3.23	11.29	3.23	0.00	0.00	8.06

[1] T—discoloured wood in the trunk; [2] B—wood in the outer part of a dead branch.

The most frequent fungal species isolated from samples with *E. parasitica* were *Eutypa* sp. 2 and *Neonectria* sp. Among the ten most frequently isolated species, only *Peniophora incarnata* was not isolated from *A. pseudoplatanus* samples where *E. parasitica* was also present. No fungal species was strictly associated with the occurrence of *E. parasitica*—all co-isolated species were also present in samples without *E. parasitica*. No significant difference was found with the Mann–Whitney U test for the Shannon diversity of fungal species between samples with and without *E. parasitica* ($p = 0.081$). Similarly, the isolated fungal community did not differ between samples with and without *E. parasitica* ($p = 0.297$), based on the results of the ANOSIM.

3.2. Colonization Rate, Relative Colonization Frequency and Density Index

Cultures were obtained from 98.5% of the investigated samples from wood of sycamore dead branches, and out of 200 samples, only three did not yield any mycelium growth on agar plates. From a total of 2700 cultured subsamples, 1744 fungal cultures were grouped into 212 morphotypes. Ninety-one morphotypes were represented by more than five cultures. Out of the 91 morphotypes, a total of 58 fungal taxa were identified. Seven out of 800 control subsamples yielded cultures, which were identified as *Eutypa* sp. 3, *Eutypa maura*, *Daldinia* sp. and *Dendryphion europaeum*.

The relative colonization frequency and density index is given in Table 6. The number of fungal taxa in each sampling site ranged from 35 to 42 (Table 7). The overall colonization rate of fungi in different sampling sites and in different isolation sources in the sample is presented in Table 7.

A high-density index was observed for *Cadophora* sp., *Eutypa lata*, *E. parasitica*, *Eutypa* sp. 2, *Fusarium lateritium* and *Neonectria* sp. (values above 3.00). Furthermore, *Fusarium avenaceum*, *Neocucurbitaria acerina*, *Coprinellus* sp. and *Nigrograna obliqua* had a high relative colonization frequency and low-density index (Table 6).

Table 6. List of taxa identified in samples from wood of dead branches of *A. pseudoplatanus* based on BLASTn queries in the NCBI, their relative colonization frequency (*F*), density index (*DI*), GenBank and the Laboratory of Forest Protection at the Slovenian Forestry Institute (ZLVG) accession numbers.

Taxon	GenBank Accession No. [1]	*F* (%)	*DI*	ZLVG No. [2]
Acremonium sp. Link	MN244544 MN240814	1.41	1.83	809 808
Alternaria sp. Nees	MN244537	0.64	1.43	781
Aureobasidium pullulans (de Bary and Löwenthal) G. Arnaud	MN244533	0.70	2.75	782
Bloxamia sp. Berk. And Broome	MN251064	0.83	2.60	810
Cadophora sp. Lagerb. And Melin	MN251055	0.90	3.50	783
Cerrena sp. Gray	MN223745	1.28	2.50	801
Clonostachys sp. Corda	MN244536	0.51	2.67	784
Coprinellus sp. P. Karst.	MN240808 MN244538 MN240810	2.75	1.79	813 812 811
Cosmospora sp. Rabenh.	MN251063	0.70	1.83	785
Cytospora sp. Ehrenb.	MN251061	0.26	1.00	786
Daldinia sp. Ces. And De Not.	MN244534 MN244541	1.28	2.50	814 815
Dendryphion europaeum Crous and R.K. Schumach.	MN251057	0.32	1.67	787
Diaporthe spp. Nitschke		4.22	2.20	
Diaporthe sp. 1	MN240809 MN244550	2.05	2.46	818 819
Diaporthe sp. 2	MN244548 MN240816	2.18	2.00	816 788
Epicoccum nigrum Link	MN216311 MN244547	2.37	2.31	820 817
Eutypa lata (Pers.) Tul. and C. Tul.	MN252417 MN252418 MN252420	2.69	3.50	821 822 824
Eutypa maura (Fr.) Sacc.	MN252421 MN252423	8.77	2.91	789 826
Eutypa spp. Tul. and C. Tul.		11.77	2.83	
Eutypa sp. 1	MN252415 MN252411	2.24	2.69	828 790
Eutypa sp. 2	MN252405	7.36	3.03	831
Eutypa sp. 3	MN252406	1.60	1.56	832
Eutypa sp. 4	MN252416	0.38	1.00	829
Eutypa sp. 5	MN252408	0.19	1.00	833
Eutypella parasitica R.W. Davidson and R.C. Lorenz	MN252407	3.97	3.26	791
Fusarium acuminatum Ellis and Everh.	MN976065	1.28	1.43	843
Fusarium avenaceum (Fr.) Sacc.	MN976063	5.50	2.00	844
Fusarium lateritium Nees	MN240811 MN976066	1.92	3.00	845 846
Fusarium merismoides Corda	MN976064	2.50	1.86	847
Nectria sp. (Fr.) Fr.	MN244545	0.38	1.50	793
Neocucurbitaria acerina Wanas., Camporesi, E.B.G. Jones and K.D. Hyde	MN216310	4.54	1.92	848
Neocucurbitaria sp. Wanas., E.B.G. Jones and K.D. Hyde	MN251052	0.45	1.40	850
Neonectria coccinea (Pers.) Rossman and Samuels	MN242704	1.92	1.58	851
Neonectria sp. Wollenw.	MN252412	3.84	3.00	795
Neosetophoma sp. Gruyter, Aveskamp and Verkley	MN244543	0.38	1.20	796

Table 6. *Cont.*

Taxon	GenBank Accession No. [1]	*F* (%)	*DI*	ZLVG No. [2]
Nigrograna obliqua Jaklitsch and Voglmayr	MN244540	2.69	1.83	878
Paraphaeosphaeria neglecta Verkley, Riccioni and Stielow	MN240812 MN244542	1.47	2.30	853 854
Parathyridaria sp. Jaklitsch and Voglmayr	MN244551	0.32	1.67	879
Penicillium brevicompactum Dierckx	MN242710	0.19	1.00	855
Peniophora incarnata (Pers.) P. Karst.	MN223746	2.82	2.00	797
Petrakia irregularis Aa	MN216309	3.58	2.80	856
Petrakia sp. Syd. And P. Syd.	MN240815	0.90	2.33	857
Phaeosphaeriaceae sp. M.E. Barr	MN251068	2.37	1.61	858
Phialemonium sp. W. Gams and McGinnis	MN251053 MN251056	0.90	1.27	860 861
Phialocephala sp. W.B. Kendr.	MN242702	1.73	1.69	862
Phomopsis pustulata (Sacc.) Died.	MN251066	3.13	2.13	799
Phomopsis velata (Sacc.) Traverso	MN244546	0.58	1.50	865
Pleosporales sp. Luttr. Ex M.E. Barr	MN251054	0.45	1.40	866
Prosthecium sp. Fresen.	MN251051	0.32	1.67	803
Pseudocosmospora rogersonii C.S. Herrera and P. Chaverri	MN242705	2.18	2.43	867
Sarocladium sp. W. Gams and D. Hawksw.	MN251067	1.22	1.36	800
Splanchnonema pupula (Fr.) Kuntze	MN251065	1.15	1.64	802
Trichoderma atroviride P. Karst.	MN242707	1.66	1.44	880
Trichoderma citrinoviride Bissett	MN242706	1.15	1.38	881
Trichoderma harzianum Rifai	MN242709	0.06	1.00	882
Trichoderma sp. Pers.	MN242708	0.13	2.00	883
Typhula sp. (Pers.) Fr.	MN251062	0.32	1.67	870
Valsa sp. Fr.	MN244549	0.38	2.00	804
Xylaria longipes Nitschke	MN251059	0.51	2.00	868
Xylaria sp. Hill ex Schrank	MN240813 MN251060	1.73	2.45	872 869

[1] Accession numbers of representative sequences deposited in GenBank. [2] Accession numbers of representative cultures deposited in the culture collection of the ZLVG.

Table 7. Total and average number of fungal taxa, colonization rates, Shannon diversity index and species evenness for different sampling sites and isolation sources.

Sampling Site	Total Number of Fungal Taxa [3]	Average Number of Fungal Taxa per Sample	Colonization Rate (%) [1]				Shannon Diversity (*H'*)	Species Evenness (*J'*)
			T	B	C	Average [2]		
Rožnik	42	4.64	92.02	100.00	2.50	96.01	3.30	0.88
Smrekovec	35	3.30	86.11	93.21	1.88	89.66	3.01	0.85
Mokrc	35	3.30	95.31	84.72	0.63	90.02	3.15	0.88
Mala voda	40	3.62	90.82	87.50	0.00	89.16	3.16	0.86
Samotorica	42	3.63	92.36	91.33	0.00	91.84	3.21	0.86

[1] T—discoloured wood in the trunk; B—wood in the outer part of a dead branch; C—visually healthy, non-discoloured wood in the trunk (control). [2] Average of T and B without C. [3] Only taxa with a frequency greater than five cultures were counted.

We compared the results about the number of identified fungal taxa from *A. pseudoplatanus* branches in foreign studies with results of our study (Table 1). In our study, 58 different fungal taxa were identified. Kowalski and Kehr [28] isolated 41 endophytic taxa in the basal part of the living branches of *A. pseudoplatanus* in Poland. In contrast, dead twigs were colonized by only 23 species in two independent studies from Poland and Germany [27,30]. The number of identified fungal taxa in our study is two times higher than the 27 taxa identified on the basis of dead branches still attached to the main stem of *A. pseudoplatanus* trees recorded by Butin and Kowalski [25]. However, we identified a higher number of taxa in comparison to other studies (Table 1). Our results on the average number of fungal taxa isolated per sample is consistent with the results of Kowalski and Kehr [28], who found that 56% of *A. pseudoplatanus* branches were colonized by three or four fungal species (Table 7).

3.3. Community Composition

Forty-three per cent of taxa were identified to the species level, and 55% to the genus level. One sequenced morphotype (1%) was identified only to the family level, and one (1%) to the order level. The level of taxon identification was conditioned with relevant BLASTn matches. Of the 58 taxa recovered from *A. pseudoplatanus*, four taxa belonged to Basidiomycota and all others to Ascomycota. Identified species were grouped into five classes, Agaricomycetes, Dothideomycetes, Eurotiomycetes, Leotiomycetes and Sordariomycetes, all belonging to Pezizomycotina and Agaricomycotina. The most frequently isolated species on *A. pseudoplatanus* samples were *Eu. Maura*, *Eutypa* sp. 2, *F. avenaceum*, *N. acerina* and *E. parasitica* (Figure 2).

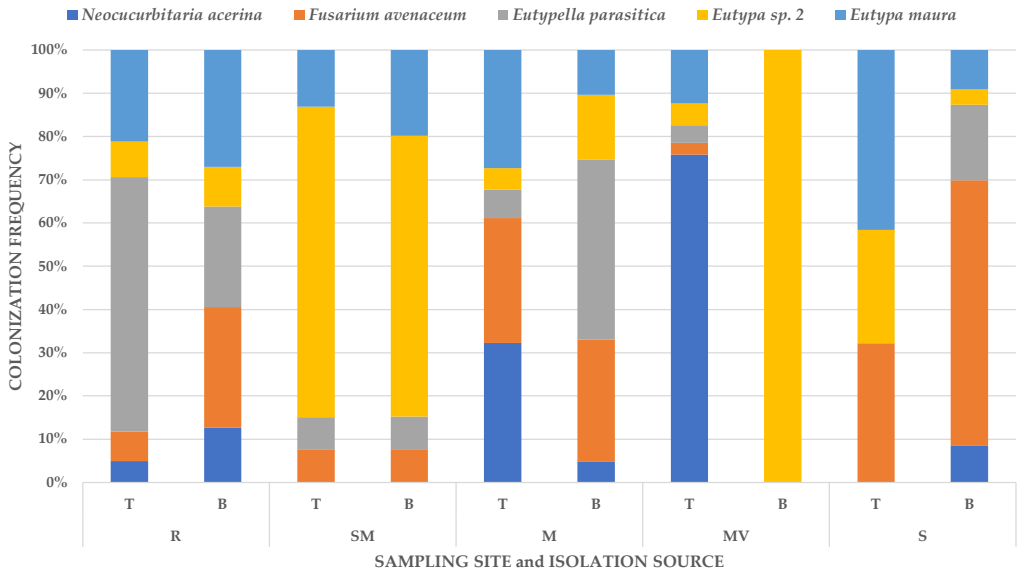

Figure 2. Colonization frequency of the five most frequently isolated fungi of wood of dead branches of young *A. pseudoplatanus* from different sampling sites and isolation sources. Sampling site: R, Rožnik; SM, Smrekovec; M, Mokrc; MV, Mala voda; S, Samotorica; Isolation source: T, discoloured wood in the trunk linked with discolouration in wood of dead branches; B, wood in outer part of a dead branch.

These taxa were isolated from an average of 6.03% of subsamples. *Neonectria* sp., *Petrakia irregularis*, *Ph. Pustulata*, *P. incarnata* and *Coprinellus* sp. were recorded in an average of 3.22% of the examined subsamples. Other taxa occurred in frequencies of less than 2.70% (Table 6). The ten most frequently isolated fungal taxa were found in almost all sampling sites, with the exception of *N. acerina*, *Neonectria* sp. (both not found in Smrekovec) and *P. incarnata* (not found in Rožnik). *Prosthecium* sp. and *D. europaeum* occurred only in Smrekovec, while *Clonostachys* sp. was found only in Rožnik. The isolated fungal community differed distinctly between the five sampling sites ($p = 0.001$).

The number of species isolated from the wood in the outer part of the dead branch (B) and discoloured wood in the trunk (T) was almost the same (52 and 55, respectively), but there were significant differences in isolated fungal species composition ($p = 0.001$). *Petrakia* sp., *Aureobasidium pullulans* and *Prosthecium* sp. were isolated only from isolation source B, while *Cadophora* sp., *Cytospora* sp. and *Penicillium brevicompactum* were specific for isolation source T. Species of *Eu. Maura*, *Eutypa* sp. 2, *N. acerina*, *E. parasitica*, *Neonectria* sp., *Ph. Pustulata* and *Coprinellus* sp. were on average two times more frequently isolated from isolation source T (63.60%) than from B (35.06%). *Petrakia irregularis* was isolated almost exclusively from B (94.64%). The frequency of *F. avenaceum* and *P. incarnata* was higher in isolation source B (average 69.13%) than in T (average 30.87%).

3.4. Species Diversity

The Shannon diversity index (H') values varied between 3.01 in Smrekovec and 3.30 in Rožnik (Table 7). No significant differences were found for the diversity of fungal species between the five sampling sites (p = 0.076). The average species evenness (J') at different sampling sites was 0.87 (Table 7). H' was higher in B than in T, but the difference was not significant (p = 0.212). Similarly, J' was higher in B than in T (Table 8). The effective number of species (ENS) ranged from 20 in Smrekovec to 27 in Rožnik. In different isolation sources, the difference in ENS was smaller (T: 33 and B: 36). To describe the beta diversity of pairs of sampling sites, we calculated the Jaccard similarity index, which uses presence–absence data and does not entail any information on the abundance of species [41]. The highest overlap (C_J = 0.65) was observed for the fungal communities in the Samotorica–Rožnik pair (Table 9). The lowest similarity between communities of fungi was observed for the Samotorica–Smrekovec pair (C_J = 0.51). Jaccard similarity index (C_J) for fungal communities between sampling sites was relatively low with an average of 0.59 (Table 9).

Table 8. Shannon diversity index (H') and species evenness (J') in different isolation sources.

	Isolation Source [1]		
	T	**B**	**C**
Shannon diversity (*H'*)	3.51	3.59	1.15
Species evenness (*J'*)	0.87	0.91	0.83

[1] T—discoloured wood in the trunk; B—wood in the outer part of a dead branch; C—visually healthy, non discoloured wood in the trunk (control).

Table 9. Jaccard similarity index (C_J) between sampling sites.

Sampling Site	Rožnik	Smrekovec	Mokrc	Mala voda
Smrekovec	0.55			
Mokrc	0.63	0.60		
Mala voda	0.62	0.51	0.60	
Samotorica	0.65	0.51	0.57	0.64

3.5. Branch Diameter and Species Diversity

The number of fungal species colonizing different branch base diameter classes ranged from 46 to 57. Differences in the average number of fungal species between different branch base diameter classes were not significant (p = 0.810). On average, 3.71 (±1.78) species of fungi were found per one sample. Branches were most frequently colonized by three (19%) or four species (22.5%). Approximately 10% of samples were colonized by a single fungal species and 17% by two species. Branches colonized by five or more fungal species represented 29.5% of investigated samples. *Fusarium avenaceum*, *N. acerina*, *Phomopsis pustulata* and *Peniophora incarnata* were the most frequent in thin branches (0.2–0.7 cm), while the other most frequently isolated species (*Coprinellus* sp., *Eu. Maura*, *Eutypa* sp. 2, *E. parasitica*, *Neonectria* sp. and *Petrakia irregularis*) were isolated mostly from branches with a 0.8–1.2 cm base diameter (Table 10). Branches in the first (0.2–0.7 cm) diameter class were most frequently colonized by *F. avenaceum*, while branches in the second (0.8–1.2 cm) and third (1.3–3.2 cm) diameter classes were usually colonized by *Eu. Maura* (Table 10).

Table 10. The most common fungal taxa in relation to branch base diameter.

Fungal Taxa	Branch Base Diameter					
	0.2–0.7 cm		0.8–1.2 cm		1.3–3.2 cm	
	n [1]	% [2]	n	%	n	%
Coprinellus sp.	6	25.00	13	54.17	5	20.83
Eutypa maura	9	19.15	25	53.19	13	27.66
Eutypa sp. 2	11	29.73	17	45.95	9	24.32
Eutypella parasitica	7	36.84	9	47.37	3	15.79
Fusarium avenaceum	20	46.51	12	27.91	11	25.58
Neocucurbitaria acerina	18	48.65	10	27.03	9	24.32
Neonectria sp.	6	30.00	8	40.00	6	30.00
Peniophora incarnata	11	50.00	5	22.73	6	27.27
Petrakia irregularis	7	35.00	9	45.00	4	20.00
Phomopsis pustulata	13	56.52	7	30.43	3	13.04
OI [3]	3.60		3.75		3.80	
Shannon diversity index (*H′*)	3.56		3.71		3.69	
Species evenness (*J′*)	0.93		0.92		0.94	

[1] Number of branches colonized; [2] shares of a specific fungal taxon in different branch base diameter classes; [3] OI—occurrence index indicating how many fungal species were found on average in samples of a given branch diameter class.

Shannon diversity index (H') values varied between 3.56 for thin branches (0.2–0.7 cm) and 3.71 for branches with a 0.8–1.2 cm base diameter (Table 10). No significant differences were found for the diversity of fungal species between the three branch base diameter classes ($p = 0.822$). Average species evenness (J') in different branch base diameter classes was 0.93 (Table 10). The effective number of species (ENS) ranged from 35 in the first (0.2–0.7 cm) branch diameter class to 40 in the second (0.8–1.2 cm) and third (1.3–3.2 cm) diameter classes. The Jaccard similarity index (C_J) for fungal communities between three branch base diameter classes was relatively high with an average of 0.80. The isolated fungal community differed distinctly between the three branch base diameter classes ($p = 0.003$), based on the ANOSIM results.

4. Discussion

Isolations from the wood of dead branches of young *A. pseudoplatanus* yielded a wide selection of fungal species. The fungus *Eutypella parasitica*, which is believed to be a non-native pathogen in Europe, was detected in all five studied sampling sites, although Eutypella cankers were observed only in three sites. The fungal communities were affected by sampling site and isolation source, but not by the presence of *E. parasitica*.

The USDA database currently lists 691 fungal species occurring on *A. pseudoplatanus* [46]. In the literature search, we found only four holistic studies of fungal species from *A. pseudoplatanus* branches [25,27,28,30], which served as a reference point for comparison with our results. The number of identified taxa in our study would have been even higher if we had also identified less frequently isolated species. It should also be noted that we identified only the species with the fastest growth and ability to grow under the employed conditions of isolation and incubation, and we can expect that the total number of fungal species present is much higher (e.g., Wu et al. [47]).

In our study, the average number of fungal species colonizing the wood of dead branches increased with increasing branch base diameter, which could be one of the indicators of branch age, thus suggesting that older branches are colonized by higher numbers of different fungal species. The most promising explanation for the non-significant differences in the average number of fungal species between different branch base diameter classes is that all examined branches were relatively uniformly thin and taken from young *A. pseudoplatanus*.

Danti et al. [48] posit that geographic origin and twig age and size, as well as different methods of surface sterilization, contribute to the differences in the number of reported fungal taxa between studies.

We believe that differences may also arise from different sources of isolation. Tedersoo et al. [49] stated that global fungal richness can be best predicted by climatic factors. A favourable climate in Slovenia is one of the possible explanations for the greater number of isolated fungal taxa compared to other studies performed in more northern latitudes such as Germany, Poland and the Czech Republic (Table 1). The detection of 58 taxa out of 91 sequenced morphotypes in five sampling sites located relatively close together (up to 26 km apart) suggests a high diversity of fungal species. Among the fungi isolated, *Eutypa maura*, *Eutypa* sp. 2, *Fusarium avenaceum*, *Neocucurbitaria acerina* and *E. parasitica* colonized more than 17% of the plated subsamples. These five species accounted for about 30% of all cultures and appear to play a dominant role in the colonization of dead samples of *A. pseudoplatanus* in our study. In comparison with other studies (Table 1), only *Eu. maura* was commonly detected, while other species were specific to our study.

The investigated samples in our study were densely colonized by a broad spectrum of fungi. In our case, almost all samples were colonized by fungi. A similar result was obtained by Kowalski and Kehr [28] in the basal parts of living branches. Fungi that colonized dead branches almost exclusively belong to Ascomycota, and to Basidiomycota in only a few cases, as already described for the living and dead basal parts of sycamore branches [25,28]. Only two taxa (*Eu. maura* and *F. avenaceum*) were present in more than 20% of samples. This is consistent with the results of Kowalski and Kehr [28].

Eutypa sp. 3, *Eu. maura*, *Daldinia* sp. and *Dendryphion europaeum* were also isolated from seven control subsamples in our study. We assume that the reason for this are endophytic life strategies of those species. Interestingly, *D. europaeum* belongs to Torulaceae, as does *Torula* sp., which was one of the most frequent taxa isolated from living branch bases in Kowalski and Kehr [28].

The species isolated and identified in our study are mainly saprotrophs on hardwood species. Identification of known pathogens of *Eutypella* and *Eutypa* is very interesting, since our study focuses on *E. parasitica*, and co-isolated species with hyperparasitic impact have the potential to be used as biological control agents. In this context, *Bloxamia* sp., *Cosmospora* sp. and *Pseudocosmospora rogersonii* are of great interest. *Pseudocosmospora rogersonii* is characterised by its mycoparasitism on *Eutypella* spp. in the USA [50]. A similar strategy has been reported for *Cosmospora* spp. Fungi in this genus parasitize other fungi, particularly species in the Xylariales [51]. Glawe [52] reported *B. truncata* on the partially decayed stromata of *Eutypella* spp. He suggested further studies to determine whether *B. truncata* is capable of mycoparasitic activity.

Eutypella parasitica was among the five most frequently isolated species in this study. It was isolated from samples acquired from all sampling sites, although the Eutypella canker of maple was observed only in Rožnik (3), Smrekovec (2) and Mala voda (1). On inventory plots in Mokrc and Samotorica, there were no cankers discovered, but those two sampling sites lie in close proximity to other sampling sites with the Eutypella canker of maple (Samotorica–Mala voda: 1680m and Mokrc–Smrekovec: 850m). We assume that the reason for the isolation of *E. parasitica* in samples from sampling sites without the Eutypella canker of maple is the wind dispersal of actively discharged ascospores from cankered trees during wet periods and possible asymptomatic infections.

In Rožnik, 45% of sampled trees were infected with *E. parasitica*. This is much higher than the usual disease occurrence, which has been estimated to be 3–5% in a stand, but similar to incidences of up to 30% recorded in a stand in the eastern part of Slovenia [21]. On average, 19% of all sampled trees in our study were infected by *E. parasitica*, which is higher than the usual incidence of 5% reported by Gross [53]. No significant difference was found for diversity ($p = 0.081$) and the fungal community ($p = 0.297$) between samples with and without *E. parasitica*, which might indicate that other fungi do not suppress or promote its growth. Furthermore, *E. parasitica* was more frequently isolated from discoloured wood in the trunks (T) than in the wood of the outer parts of dead branches (B). It is likely that *E. parasitica* in the wood of the outer parts of dead branches (B) is overgrown or replaced by other fungi, and because of strong competition, the fungus quickly progresses into the wood of the trunk. Based on the results of isolations and maple inventory, it is likely that *E. parasitica* has an even wider distribution than previously thought. We assume that the disease was simply overlooked

previously, since there was no systematic monitoring in those sampling sites and young infections are very inconspicuous [14], or *E. parasitica* is capable of causing asymptomatic infections.

In addition to *E. parasitica*, different species of *Eutypa* spp. were also identified. *Eutypa* spp. were among the most frequently isolated taxa in our study. They were identified in 23% of plated subsamples. Fungi from the genus *Eutypa* were represented by *Eu. maura* and *Eu. lata*, two well-known species from woody tissues of trees, and five other species not identified to the species level. Unterseher and Tal [54] reported *Eu. maura* as a dominant component of dead twigs and branches. In their study the stromata of *Eu. maura* covered most of the branch surface and probably made it impossible for saprophytic secondary invaders to successfully colonize the substrate. This could also be a possible explanation for our results. *Eutypa lata* is a worldwide pathogen of many woody plants [55–57]. It is a well-known cause of one of the most destructive diseases of *Vitis vinifera* L.—Eutypa dieback or dead-arm disease of grapevine [55,58]. Rappaz [59] found a host specific variety of *Eu. lata* on the wood and bark of *A. campestre* L. in France and *A. pseudoplatanus* in Switzerland. The old name (*Eu. lata* var. *aceri* Rappaz) has now been changed to *Eu. lata*, after Index Fungorum [60].

Our results suggest high fungal species diversity in wood of dead branches of *A. pseudoplatanus*. The Shannon diversity index (*H′*) for fungal species from different sampling sites ranged from 3.01 to 3.30 ($p = 0.076$), from 3.51 to 3.59 in different isolation sources ($p = 0.212$) and from 3.56 to 3.71 in different branch base diameter classes ($p = 0.822$). These values are consistent with Magurran [41], who stated that typical values of *H′* lie between 1.5 and 3.5, and only rarely exceed four. Gennaro et al. [61] reported Shannon diversity indices in the range between 0.21 and 0.80 for endophytic fungi from different tissues of healthy and declining *Quercus robur* L. and *Q. cerris* L. in Italy. Hanácková et al. [62] found significantly higher diversity in the winter shoots of *Fraxinus excelsior*. The Shannon diversity index of endophytic fungi from *Ulmus macrocarpa* Hance, *Q. liaotungensis* Koidz. and *Betula platyphylla* Sukaczev ranged from 1.28 to 2.11 [63]. Therefore, this comparison suggests a relatively high diversity of fungal taxa in our study. The above authors also detected differences in diversity between different tissue types. In general, the similarity between communities of different sampling sites was relatively low, with an average of 0.59. Kowalski et al. [64], for example, found higher values of similarity between fungal communities on the living and dead stems and twigs of *F. excelsior* (ranging from 0.65 to 0.92).

The isolated fungal community differed distinctly between the five sampling sites ($p = 0.001$), between the different isolation sources ($p = 0.001$), and between the different branch base diameter classes ($p = 0.003$). The fungal community structure of *A. pseudoplatanus*-dead branches in our study could have been affected by the decay rate of the samples, age of trees and branches, season of sampling and overall tree health status, as already reported by Gennaro, Gonthier and Nicolotti [61] and Hanácková, Havrdová, Černý, Zahradník and Koukol [62]. The distribution and diversity of fungal species is also dependent on environmental factors, such as temperature, rainfall, tree composition, water availability and soil characteristics [30,54,64]. The degree of colonization may also be dependent on the plant community and branch diameter [28]. Danti, Sieber and Sanguineti [48] and Kowalski, Kraj and Bednarz [64] suggested the dependency of observed species composition and frequency on the method of isolation. The authors pointed out the possibility that the method of isolation used does not yield a complete picture of the real number and frequency of species. This could be also the case in our study. The observed fungal community could have also been a consequence of the generalized incubation conditions. However, we are aware that only the fastest growing and the most frequently isolated culturable fungal species were identified, and that there are many other species left to be identified. Species classified as "sp." and identified only to genus or an even higher level would need further morphological and molecular analyses.

5. Conclusions

Isolations from the wood of dead branches of young *A. pseudoplatanus* yielded 1744 fungal cultures, which were grouped into 212 morphotypes. Fifty-eight fungal taxa were identified from morphotypes

represented by more than five cultures. The most frequently isolated species were *Eutypa maura*, *Eutypa* sp. 2, *Fusarium avenaceum*, *Neocucurbitaria acerina* and *Eutypella parasitica*. Since there was no significant difference in the fungal community of samples with or without *E. parasitica*, we assume that *E. parasitica* did not have a strong impact on the success of tissue colonization with other isolated species. On the other hand, the overall fungal communities of samples were affected by the sampling site, isolation source and branch base diameter. In contrast, branch thickness did not prove to be a significant factor in the fungal species diversity of the dead branches of *A. pseudoplatanus* in our study because they were relatively thin and young. The most interesting finding of our research is the isolation of *E. parasitica* from all five investigated sampling sites, although Eutypella cankers were observed only in three, indicating the possibility of asymptomatic infection and the long-distance wind dispersal of its ascospores.

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