UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Ana BRGLEZ

ANTAGONIZEM MED GLIVO *Eutypella parasitica* IN IZBRANIMI VRSTAMI GLIV V LESU ODMRLIH VEJ GORSKEGA JAVORJA (*Acer pseudoplatanus*)

DOKTORSKA DISERTACIJA

Ljubljana, 2022

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ANTAGONISM BETWEEN THE FUNGUS *Eutypella parasitica* AND SELECTED FUNGAL SPECIES IN DEAD BRANCHWOOD OF SYCAMORE MAPLE (*Acer pseudoplatanus*)

DOCTORAL DISSERTATION

Ljubljana, 2022

Na podlagi Statuta Univerze v Ljubljani in po sklepu Senata Biotehniške fakultete ter sklepa 24. seje Komisije za doktorski študij Univerze v Ljubljani z dne 4. 2. 2020 (po pooblastilu 6. seje Senata Univerze v Ljubljani z dne 27. 3. 2018) je bilo potrjeno, da kandidatka Ana Brglez izpolnjuje pogoje za opravljanje doktorata znanosti na bolonjskem Interdisciplinarnem doktorskem študiju Bioznanosti, znanstveno področje upravljanje gozdnih ekosistemov. Za mentorja je bil imenovan višji znan. sod. dr. Nikica Ogris.

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AI *Eutypella parasitica*, povzročiteljica javorovega raka, uničujoče bolezni javorjev v Evropi in Severni Ameriki, najpogosteje okuži deblo gostitelja skozi odmrle veje ali rane v skorji. Zaradi morebitnega vpliva glivne združbe na okužbo in rast *E. parasitica* smo proučili vrstno sestavo gliv v lesu odmrlih vej gorskega javorja (*Acer pseudoplatanus*). Glive smo izolirali v čiste kulture iz različnih mest v odmrli veji. Najpogosteje smo izolirali *Eutypa maura*, *Eutypa* sp., *Fusarium avenaceum*, *Neocucurbitaria acerina* in *E. parasitica*. Ugotovili smo neznačilne razlike v pestrosti glivnih vrst in značilne razlike v glivnih združbah med različnimi lokacijami, med različnimi mesti izolacije in med različnimi debelinami vej. Analizirali smo vpliv najpogostejših vrst gliv v lesu odmrlih vej gorskega javorja na rast *E. parasitica* v čisti kulturi in s testom mini-blok ugotavljali njihov vpliv na razkroj lesa naših najpogostejših vrst javorjev. Na podlagi izračuna antagonističnega indeksa in uspešnosti reizolacij iz interakcijske cone smo *E. parasitica* šteli za šibkejšo tekmovalno vrsto. Izolati *Eutypa* sp., *Eu. maura*, *Neonectria* sp. in *Peniophora incarnata* so se izkazali za najučinkovitejše inhibitorje *E. parasitica*, ki bi jih lahko potencialno uporabili tudi za zatiranje *E. parasitica*. S testom mini-blok smo ugotovili relativno počasen razkroj lesa zaradi delovanja *E. parasitica*. V primerjavi z izolatoma znanih trohnobnih gliv je povzročila značilno manjše izgube mase. Na podlagi mikroskopije in obarvanja vzorcev sklepamo, da *E. parisitica* povzroča belo trohnobo. Z raziskavo smo podrobneje raziskali določene lastnosti *E. parasitica* in pri tem odprli številna nova vprašanja.

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AB *Eutypella parasitica*, the causative agent of Eutypella canker of maple, a destructive disease of maples in Europe and North America, most frequently 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*, we examined the species composition of fungi colonizing the wood of the dead branches of sycamore maple (*Acer pseudoplatanus*). Isolations were made from different isolation sources in a dead branch. The most frequently isolated species were *Eutypa maura*, *Eutypa* sp., *Fusarium avenaceum*, *Neocucurbitaria acerina*, and *E. parasitica*. We did not detect differences in species diversity, but fungal communities differed between sampling sites, between isolation sources, and between branch thickness classes. We tested the most frequently isolated fungi from the wood of dead branches of sycamore maple in dual cultures with *E. parasitica* and determined their effect on wood decay in our most common maple species using a mini-block test. Based on the calculation of an index of antagonism and re-isolation success from the interaction zone, we consider *E. parasitica* to be a weak competitor. *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *Peniophora incarnata* have been shown to be the most effective inhibitors of *E. parasitica*. Relatively slow wood decay due to the action of *E. parasitica* was detected by the mini-block test. Compared to isolates of known decay fungi, it resulted in significantly lower weight loss. Based on microscopy and staining of the samples, we conclude that *E. parasitica* could be considered to cause white rot. We have examined certain areas of *E. parasitica* biology and raised several new questions.

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OKRAJŠAVE IN SIMBOLI

AI	antagonistični indeks
CODIT	kompartmentalizacija razkroja v drevesu (angl. *Compartmentalization Of Decay In Trees*)
DSMZ	nemška zbirka mikroorganizmov in celičnih kultur (nem. *Deutsche Sammlung von Mikroorganismen und Zellkulturen*)
EF-1α	elongacijski faktor 1-alfa (angl. *elongation factor 1-alfa*)
ITS	notranji prepisni vmesnik (angl. *internal transcripted spacer*)
LM	svetlobna mikroskopija (angl. *light microscopy*)
MEA	gojišče z ekstraktom ječmenovega slada (angl. *malt extract agar*)
PDA	krompirjev agar z glukozo (angl. *potato dextrose agar*)
rDNA	ribosomska deoksiribonukleinska kislina
s	uspešnost reizolacij iz interakcijske cone
SEM	vrstična elektronska mikroskopija (angl. *scanning electron microscopy*)
ZIM	Zbirka industrijskih mikroorganizmov
ZLVG	Zbirka živih kultur Laboratorija za varstvo gozdov
ZGS	Zavod za gozdove Slovenije

1 UVOD

1.1 PREDSTAVITEV RAZISKOVALNEGA PROBLEMA

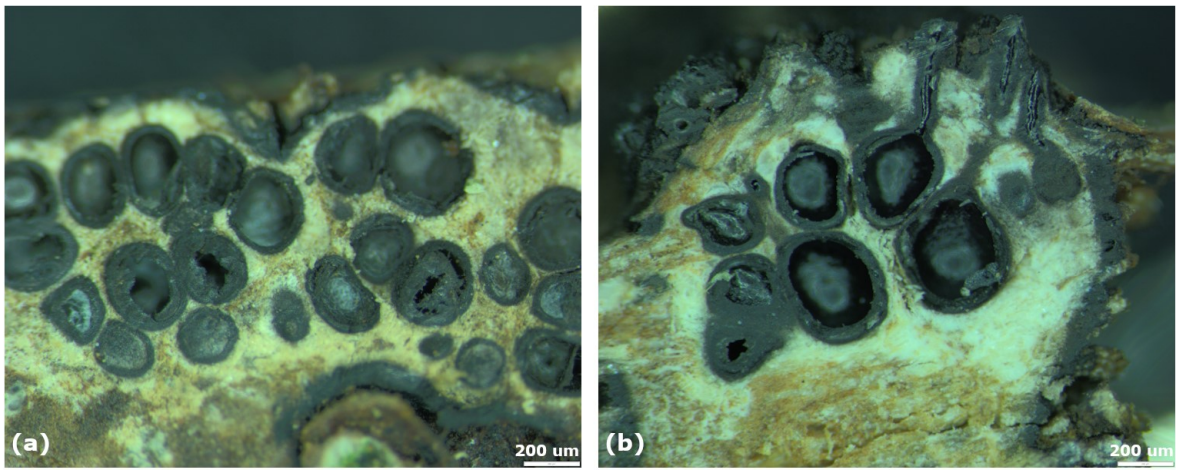
Gozdna fitopatologija je veda o boleznih gozdnega drevja, ki jih povzročajo abiotski in biotski dejavniki, kot so glive, bakterije, fitoplazme, virusi, parazitske cvetnice ipd. (Maček, 2008). Beseda fitopatologija je sestavljena iz grških besed: *phyton* (rastlina), *pathos* (bolezen) in *logos* (veda). Glavna naloga fitopatologije je spoznavati povzročitelje obolenj, njihov način življenja in razvojni krog ter najti morebitne načine za zatiranje bolezni (Maček, 2008). Rastlinske bolezni so okuževale rastline že v pradavnini (Maček, 2008), pojavi novih oz. tujerodnih škodljivih organizmov pa se v zadnjih desetletjih hitro širijo zaradi podnebnih sprememb, globalne trgovine, povečanega prometa in turizma (Hulme in sod., 2009; MacLeod in sod., 2010; Seebens in sod., 2015). S človekovo pomočjo tujerodne vrste lažje dosežejo nova območja in se širijo na daljše razdalje.

1.1.1 *Eutypella parasitica* in javorov rak

Ena izmed tujerodnih vrst gliv, ki je bila vnesena v evropski prostor, kjer se je tu tudi uspešno udomačila, je *Eutypella parasitica* R. W. Davidson & R. C. Lorenz. Glivo so odkrili in opisali leta 1937 v okolici Velikih jezer, na meji med Kanado in Združenimi državami Amerike (Davidson in Lorenz, 1938). Vrsta okužuje vse vrste javorjev (*Acer* spp.) in povzroča bolezen javorov rak. V Ameriki se pojavlja na sladkornem (*Acer saccharum* Marsh.) in rdečem javorju (*A. rubrum* L.), redkeje tudi na ameriškem (*A. negundo* L.), ostrolistnem (*A. platanoides* L.), srebrnem (*A. saccharinum* L.), črnem (*A. nigrum* Mich.), gorskem (*A. pseudoplatanus* L.), progastem (*A. pennsylvanicum* L.) in oregonskem javorju (*A. macrophyllum* Prush.) (Davidson in Lorenz, 1938; French, 1967; Kliejunas in Kuntz, 1972, 1974). Javorov rak je bil v Sloveniji in hkrati tudi v Evropi prvič zabeležen leta 2005 (Jurc in sod., 2006). Pri nas sta najpogosteje okužena gorski javor in maklen (*A. campestre* L.), bolezen pa se pojavlja tudi na ostrolistnem javorju in drugih vrstah javorjev (Ogris in sod., 2022). *Eutypella parasitica* je taksonomsko uvrščena med zaprtotrosnice (Ascomycota), poddeblo Pezizomycotina, razred Sordariomycetes, podrazred Xylariomycetidae, red Xylariales in družino Diatrypaceae (Index Fungorum, 2009).

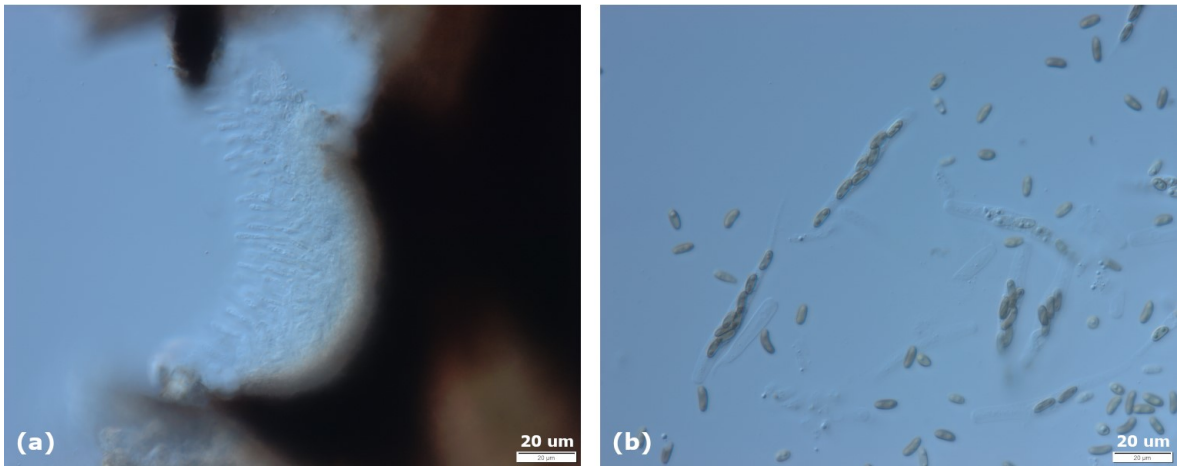
Gliva *E. parasitica* najpogosteje okuži gostitelja skozi odmrlo vejo ali poškodbo na deblu (French, 1967). Pri tem starost gostitelja nima pomembne vloge (French, 1967), čeprav Davidson in Lorenz (1938) v prvem opisu bolezni navajata, da lahko gliva okuži gostitelja le v mladosti. French (1967) s svojo raziskavo ni ugotovil enotne razporeditve okužb po debelinskih razredih gostiteljskih dreves. Prav tako tudi ni odkril povezave med deležem gostiteljskih dreves v sestoju in stopnjo okuženosti (French, 1967). Navadno je v sestoju okuženih 3–5 % javorjev (ponekod celo do 50 %) (Ogris in sod., 2009), kar je glede na infekcijski potencial vrste relativno malo (French, 1967). Po podatkih raziskave, ki so jo opravili Ogris in sod. (2009), se namreč na eni rakavi rani v povprečju razvije 647.000

peritecijev (Slika 1), ki v ugodnih razmerah lahko v eni uri sprostijo približno eno milijardo spor. Optimalna temperatura za rast glive je 24–28 °C (French, 1967; Johnson in Kuntz, 1979). Za sproščanje spor imata poleg ugodne temperature pomembno vlogo tudi zadostna količina padavin in zračna vlaga (French, 1967). Lachance (1971) navaja, da je za sproščanje askospor potrebnih vsaj 2,5 mm padavin na m^2, ki zadostno navlažijo skorjo s periteciji, in temperatura zraka več kot 4 °C. Visoka zračna vlažnost vpliva na počasnejše sušenje skorje s periteciji in podaljša obdobje sproščanja spor po dežju, neposredno pa ne vpliva na sam začetek sproščanja. Največje količine se sprostijo v šestih urah po navlažitvi (Johnson in Kuntz, 1979). Temperatura ni kritičen dejavnik za sproščanje spor, vpliva pa na absorpcijo vode v stene peritecijev. Pri višji temperaturi se higroskopne stene peritecijev hitreje navlažijo, nabreknejo in izvržejo aske z askosporami (French, 1967) (Slika 2). Z oddaljevanjem od vira se hitro zmanjšuje število spor (Johnson in Kuntz, 1979). Večina novih okužb se pojavlja v skupinah okoli izvornih rakov (Kliejunas in Kuntz, 1974; Martinez in sod., 2003; Ogris in sod., 2009). Pogosteje se raki razvijejo na podstojnem drevju (Kliejunas in Kuntz, 1974). Vzroki za maloštevilne okužbe še niso raziskani. Eden izmed morebitnih vzrokov je nizka stopnja virulentnosti, ki *E. parasitica* uvršča med šibke patogene. Drugi je način izmetavanja askospor, ki iz peritecijev izhajajo v skupinah po osem, imajo relativno veliko maso, zato padejo na tla večinoma v radiju 25 m od okuženega drevesa (Johnson in Kuntz, 1979). Tretji, še ne raziskan vzrok redkih okužb pa so domnevno združbe gliv, ki se pojavljajo v odmrlih vejah javorjev. Vrste med seboj tekmujejo in vplivajo na uspešnost naselitve in izražanja bolezenskih znakov (Porter in Carter, 1938; Holmer in sod., 1997; Schmit, 1999; Rezgui in sod., 2018). Morda je *E. parasitica* šibek patogen z nizko stopnjo virulentnosti, ki ga hitro izločijo oz. nadomestijo druge, tekmovalno uspešnejše vrste.



Slika 1: Prečni (a) in vzdolžni (b) prerez skozi lumne peritecijev (Foto: A. Brglez).

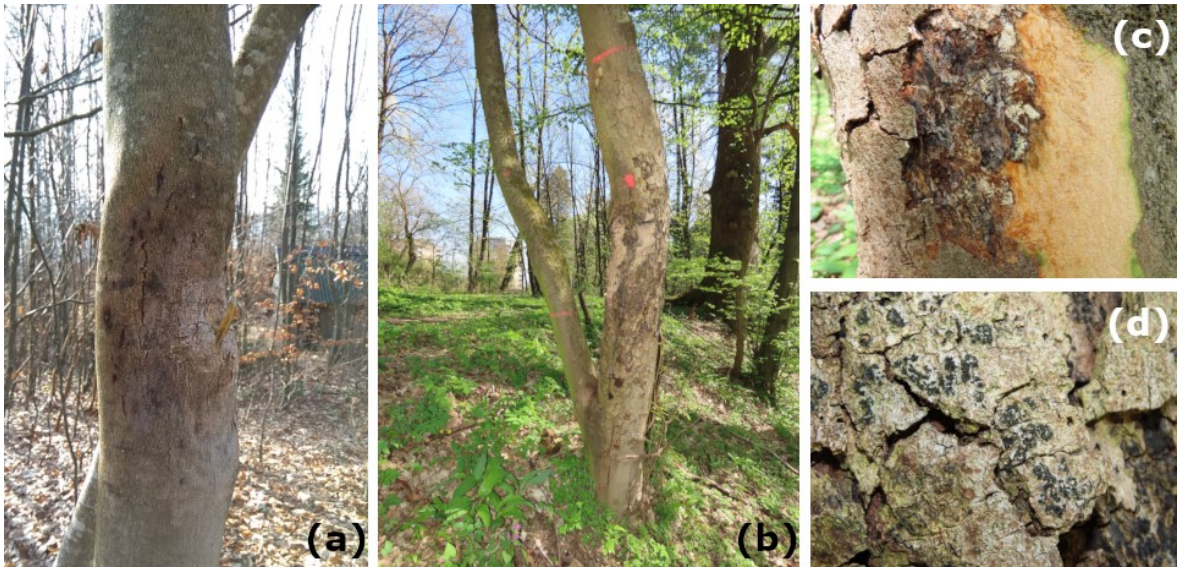
Figure 1: Cross (a) and longitudinal (b) section through the lumens of the perithecia (Photo: A. Brglez).



Slika 2: Mikroskopske značilnosti javorovega raka: a) nastajajoči aski in b) sproščeni aski z askosporami (Foto: A. Brglez).

Figure 2: Microscopic characteristics of Eutypella cancer of maple: a) asci formation, and b) released asci with ascospores (Photo: A. Brglez).

*Eutypella parasitic*a se na krajših razdaljah razširja z vetrom, ki prenaša askospore iz peritecijev. Na daljših razdaljah pa se bolezen širi s trgovino z lesom in okrasnim drevjem (EPPO, 2008). Na deblih javorjev povzroči razvoj pravilne eliptične rakave rane (Slika 3, a in b) (Ogris in sod., 2009), kar je posledica hitrejše rasti glive v vertikalni kot lateralni smeri (French, 1967). Navadno je na posameznem okuženem deblu le ena rakava rana (Davidson in Lorenz, 1938). Skoraj vedno so v središču raka opazni ostanki odmrle veje, skorja je rahlo ugreznjena, v skorji se razrašča belo do bež podgobje (micelijske pahljačice) (Slika 3, c) (French, 1967). Deblo je deformirano, odmrla skorja pa ostane na deblu pritrjena več let zaradi močnega podgobja. Na osrednjem delu rane se okoli sedem let po okužbi pojavijo črna trosišča v obliki peritecijev z dolgimi črnimi vratovi, ki jih zaradi velikega števila opazimo na površini rane (Slika 3, d) (Davidson in Lorenz, 1938). Večina peritecijev nastane v rastni sezoni (Kliejunas in Kuntz, 1972). Zreli periteciji v povprečju v premeru merijo 600–1000 µm, medtem ko so njihovi vratovi lahko dolgi tudi do 5 mm (Lachance in Kuntz, 1970). Gliva se razrašča v skorji in lesu, ki ga razgrajuje. V lesu je rast hitrejša, zato je poškodba večja, kot je videti po velikosti raka od zunaj (Ogris in sod., 2009). Sinclair in sod. (1987) navajajo, da gliva zraste od 1 do 2 cm na leto. Okužena mlajša drevesa navadno hitro propadejo, še preden bi se razvili očitni simptomi bolezni. Najočitnejši simptom zgodnjih okužb so micelijske pahljačice, uleknjena skorja in manjša odebelitev na mestu okužbe (French, 1967).



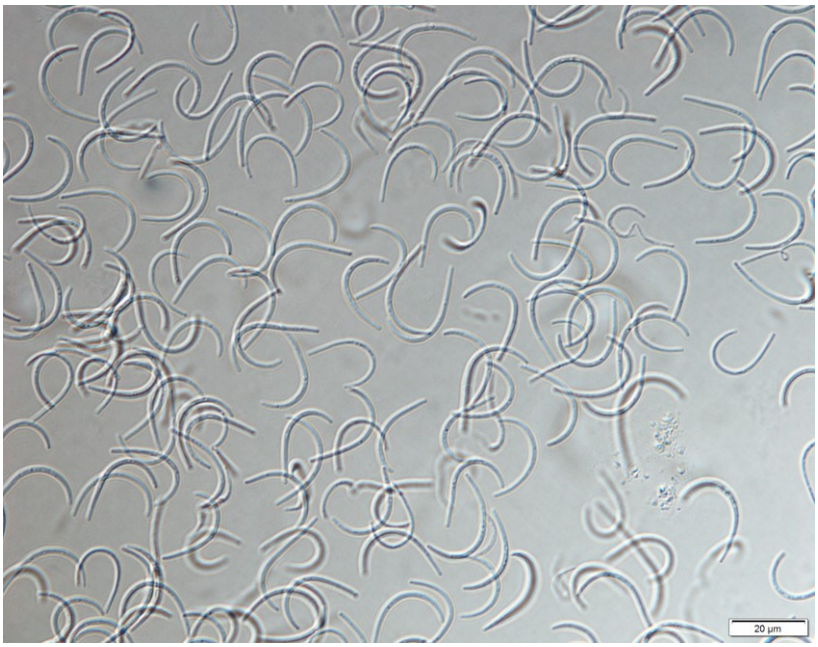
Slika 3: Značilni simptomi javorovega raka: a) uleknjena skorja z odmrlo vejo v središču raka, b) eliptična rakava rana, c) micelijske pahljačice v skorji in d) periteciji (Foto: A. Brglez).

Figure 3: Typical symptoms of Eutypella canker of maple: a) depressed bark with dead branch stub in the center, b) elliptical shape of the canker, c) mycelial fans in the bark, and d) perithecia (Photo: A. Brglez).

Bolezen povzroča relativno veliko ekološko in ekonomsko škodo (Gross, 1984b; Martinez in sod., 2003). Poškodovani del debla z rakavo rano je namreč tehnično razvrednoten. Mlajša drevesa zaradi okužbe navadno propadejo, medtem ko lahko starejša z glivo rastejo več desetletij. Ker gliva razkraja tudi les, so okuženi javorji slabše mehansko stabilni in občutljivejši za veter, sneg in žled (Davidson in Lorenz, 1938; Ogris in sod., 2005). Krošnje okuženih dreves sicer niso očitno prizadete, občasno odmrejo le posamične veje. Pod rakavo rano se po navadi pojavijo tudi številni adventivni poganjki (French, 1967). Po raziskavah se 60 % okužb pojavi do višine 244 cm, samo slabih 20 % rakov se razvije višje od 488 cm (French, 1967). Bolezen se širi počasi in prav ta lastnost omogoča, da lahko precej uspešno zaustavljamo njeno širjenje.

Davidson in Lorenz (1938) sta prva opisala tudi rast *E. parasitica* v kulturi. Gliva dobro raste na različnih hranilnih medijih in razvije gost bel micelij, ki sčasoma postaja bež do kremaste barve. Povprečna hitrost rasti je 20 mm v sedmih dneh pri temperaturi 25 °C (Davidson in Lorenz, 1938), 58 mm v sedmih dneh pri temperaturi 28 °C (French, 1967) oz. 2,4 mm na dan pri 24 °C (Ogris in sod., 2009). Raziskave so pokazale dobro rast *E. parasitica* med 20 in 35 °C (Davidson in Lorenz, 1938). French (1967) kot optimalno temperaturo za rast glive v kulturi navaja 28 °C. Kulture potrebujejo približno štiri do šest tednov inkubacije pri temperaturi 25 °C, da začnejo proizvajati spore v piknidijem podobnih strukturah (Davidson in Lorenz, 1938; French, 1967; Glawe, 1983; Sinclair in sod., 1987). Raziskovalci si niso enotni glede taksonomske uvrstitve nespolne oblike glive. Davidson in Lorenz (1938) sta nespolni stadij opisala kot *Libertella* sp., ki oblikuje konidije neposredno na miceliju, medtem ko French (1967) nespolno obliko *E. parasitica* uvršča med *Cytosporina* sp., ki v

kulturi oblikuje piknidije s konidiji v obliki vitic jantarne barve. Konidiji za samo razširjanje glive niso pomembni (Slika 4).

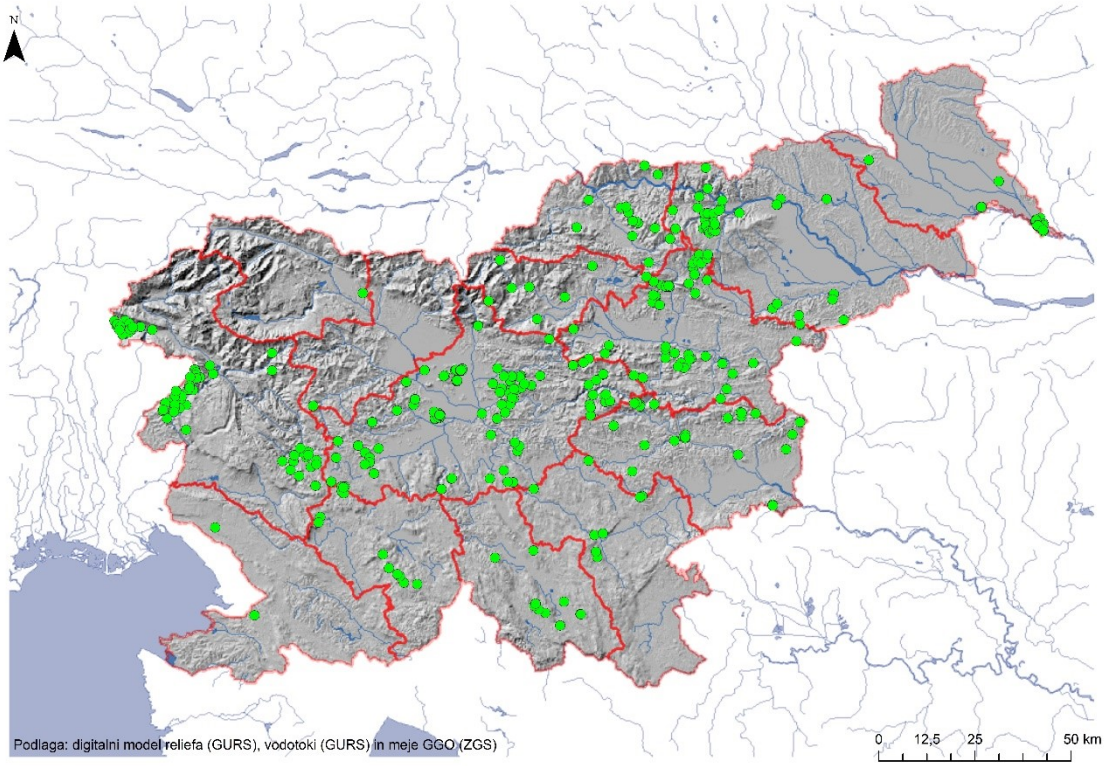


Slika 4: Nespolni trosi (konidiji) iz kulture *E. parasitica* (Foto: A. Brglez).

Figure 4: Asexual spores (conidia) from *E. parasitica* culture (Photo: A. Brglez).

V Sloveniji so glivo *E. parasitica* odkrili leta 2005 (Jurc in sod., 2006). Ogris in sod. (2006) so nato na podlagi primernosti podnebnih razmer in prisotnosti gostiteljskih dreves razvili model tveganja za širjenje javorovega raka po Evropi; 13 % površine so uvrstili med območja z najvišjo stopnjo tveganja. Kot najbolj ogrožena območja so navedli Slovenijo, Bosno in Hercegovino, Srbijo, Makedonijo, Hrvaško, Apenine, osrednje Pireneje, Češko, jug Slovaške, Avstrijo, osrednjo in južno Nemčijo, Poljsko, sever Švice ter vzhod Francije in Ukrajine. Rezultati modela se dobro ujemajo s poznejšimi najdbami javorovega raka v evropskih državah: Avstrija (Cech, 2007), Hrvaška (Ogris in sod., 2008), Nemčija (Cech in sod., 2016), Madžarska (Jurc in sod., 2016), Poljska (Černý in sod., 2017), Češka (Černý in sod., 2017), Italija (Bregant, 2018; Jurc in sod., 2019) in Švica (Dubach in sod., 2022). Poleg modela, ki ocenjuje tveganje na nivoju celotne Evrope, je nastal tudi model širjenja javorovega raka v Sloveniji (Ogris in sod., 2007). Ocena tveganja je bila narejena za takratne razmere in tri izbrane scenarije podnebnih sprememb v dveh časovnih obdobjih. Napoved je temeljila na poznavanju občutljivosti različnih vrst javorjev, razmnoževanja in širjenja glive v povezavi z arealom gostiteljskih dreves in primernosti podnebja. Za takratne podnebne razmere je bilo 8 % gozdov Slovenije ocenjenih z visokim tveganjem, 43 % s srednjim, 44 % z majhnim in 4 % gozdov z zelo majhnim tveganjem, da se pojavi javorov rak. Raziskovalci so ugotovili, da se bo v prihodnje delež površin z javorovim rakom verjetno manjšal oz. bo večji delež površin vključen v nižjo stopnjo tveganja zaradi predvidenega zmanjševanja lesne zaloge javorjev v gozdnih sestojih zaradi podnebnih sprememb. V

Sloveniji je bilo 25. 3. 2022 potrjenih 480 okužb z *Eutypella parasitica* (Ogris in sod., 2022) (Slika 5).



Slika 5: Razširjenost potrjenih okužb z *Eutypella parasitica* v Sloveniji 25. 3. 2022 (Ogris in sod., 2022).

Figure 5: Prevalence of confirmed *Eutypella parasitica* infections in Slovenia on 25.3.2022 (Ogris in sod., 2022).

V Sloveniji je po podatkih Zavoda za gozdove Slovenije (ZGS) za leto 2021 lesna zaloga javorjev znašala 11,9 milijona m^3. Od tega je bilo 96,3 % gorskega javorja, 1,6 % maklena in 1,3 % ostrolistnega javorja (ZGS, 2022). V rodu javorjev je sicer na severni polobli znanih 150–200 vrst, v Sloveniji pa jih samoniklo raste šest: gorski, ostrolistni, poljski, trokrpi, topokrpi in tatarski javor. V Evropi je najpogostejši gorski javor, ki izvira z gorskih predelov srednje Evrope (Spiecker in sod., 2009). Areal gorskega javorja se razteza od Turčije in Španije na jugu do Irske in Švedske na severu (Rusanen in Myking, 2003). Odlikuje ga dobra prilagodljivost na raznolike rastiščne razmere (Hein, 2009). Ostrolistni javor je domoroden na območju vse od Grčije, severne Italije in Pirenejev do juga skandinavskih držav (Caudullo in de Rigo, 2016). Razširjen je tudi v Mali Aziji in na Kavkazu (Brus, 2008). Naravno območje razširjenosti poljskega javorja oz. maklena obsega večino Evrope; raste v pasu od Pirenejev, Sicilije, Grčije in severne Turčije do srednje Anglije, južne Švedske in Danske. Posamezne primerke je mogoče najti tudi v Španiji in severni Afriki (Nagy in Ducci, 2004). Trokrpi javor je naravno razširjen po skoraj celotnem Sredozemlju do Male Azije in severne Afrike (Bavcon in sod., 2011). Topokrpi javor je endemit balkanskega visokega krasa, naravno je razširjen v gozdovih na Dinarskem gorovju na vsem Balkanskem polotoku (Brus,

2008). Tatarski javor pa je naravno razširjen v vzhodni Evropi in zahodni Aziji. V Sloveniji so ga v naravi zadnjikrat zanesljivo našli leta 1907, dandanes pa ne vemo več za nobeno njegovo naravno rastišče (Brus, 2008).

1.1.2 Združbe gliv, ki se pojavljajo na odmrlih vejah gorskega javorja

Glive so druga vrstno najbogatejša skupina organizmov (Purvis in Hector, 2000). Wu in sod. (2019) ocenjujejo, da je skupno število glivnih vrst od 11,7 do 13,2 milijona. Ocena je precej višja od začetne hipoteze o 1,5 milijona (Hawksworth, 1991) in 2,2–3,8 milijona (Hawksworth in Lücking, 2017) gliv. Doslej je bilo opisanih samo okoli 7 % vrst (Wu in sod., 2019), kar kaže, da je na tem področju še veliko neraziskanega.

Glive so torej precej razširjena skupina organizmov. Večina je saprofitov in razgrajujejo mrtve organske snovi. Preostale glive so specializirane za okužbe živih tkiv in jih delimo na patogene in endofite (Sieber, 2007). Meja med njimi je pogosto zabrisana (Bayman, 2007). Endofiti so še posebno zanimivi, saj povsem asimptomatsko naselijo gostiteljevo tkivo. Na tak način lahko živijo ves čas ali pa po določeni inkubacijski ali latentni dobi in v ugodnih razmerah okolja (tj. v gostitelju, katerega metabolizem se je spremenil zaradi določenih stresnih dejavnikov) spremenijo svojo življenjsko strategijo (Bayman, 2007; Sieber, 2007). Takšna primera sta *Cryptostroma corticale*, ki povzroča sajasto odmiranje skorje (Ogris in sod., 2021), in *Biscogniauxia nummularia*, ki povzroči pooglenitev bukve (Luchi in sod., 2015). Splošno mnenje je, da so rastline brez endofitov izjemno redke (Gennaro in sod., 2003). Interakcije endofitov in njihovih gostiteljev so zapletene, večplastne in se lahko spreminjajo skozi čas. Večina rastlin ima izjemno pestro združbo endofitov, ki vključuje nekaj dominantnih in veliko redkih vrst. Endofiti istega gostitelja se lahko občutno razlikujejo med posameznimi lokacijami (Bayman, 2007). Življenjski slog (mutualizem, komenzalizem, parazitizem) večine endofitov ni znan (Sieber, 2007).

Združbe gliv z različnimi življenjskimi strategijami, ki se pojavljajo na odmrlih vejah gorskega javorja, so slabo raziskane. Glive, ki naseljujejo odmrle veje in sodelujejo pri razkroju lesa, večinoma spadajo v debla Ascomycota in Deuteromycota (Butin in Kowalski, 1986) ter so navadno že pred odmrtjem veje v skorji in lesu na endofiten način (Maček, 2008; Parfitt in sod., 2010). Pri pregledu literature smo zasledili nekaj študij, v katerih so raziskovali glive na gorskem javorju: Butin in Kowalski (1986) – odmrle veje, Chlebicki (1988) – les odmrlih vej, Kowalski in Kehr (1992) – dnišča živih vej, Unterseher in sod. (2005) – odmrle veje v krošnji in Johnova (2009) – razkrajajoča debla, panji, poganjki in veje. Obstajajo tudi raziskave glivnih asociacij na rdečem (*A. rubrum*) (Green in sod., 1981), sladkornem (*A. saccharum*) (Yang, 2005), ostrolistnem (*A. platanoides*) (Terho in Hallaksela, 2008) in mandžurskem javorju (*A. ginnala*) (Qi in sod., 2009) ter *A. truncatum* (Sun in sod., 2011). V literaturi zasledimo tudi primere raziskovanja glivnih združb na listih, iglicah, vejah in deblih drugih drevesnih vrst; le-ti so nam služili kot dodaten vir

metodoloških idej za našo raziskavo (Boddy in sod., 1987; Sieber, 1988; Fisher in Petrini, 1990; Sieber in sod., 1995; Danti in sod., 2002; Gennaro in sod., 2003; Sun in sod., 2012; Kowalski in sod., 2016; Hanácková in sod., 2017). Manjka pa podrobna študija gliv v lesu odmrlih vej gorskega javorja v povezavi z *E. parasitica*.

Da bi ugotovili pestrost glivnih vrst v različnih substratih, se raziskovalci tradicionalno poslužujejo morfoloških metod z dodatno uporabo v zadnjem času hitro napredujočih in razvijajočih se molekularnih tehnik. Zaradi časovne komponente in relativno enostavnejšega pristopa, ki ne terja poglobljenega mikološkega znanja, je v zadnjem času molekularni pristop privlačnejši. Številne raziskave na tem področju omogočajo hitro in enostavno identifikacijo organizmov. Takšen primer so recimo okužbe z *E. parasitica*, za katere sta bila razvita vrstno specifična oligonukleotidna začetnika (angl. primer) EpF in EpR, ki omogočata hitro in enostavno molekularno identifikacijo vrste (Piškur in sod., 2007). Omenjene molekularne metode smo uporabljali tudi v sklopu naših raziskovalnih ciljev.

1.1.3 Konkurenčnost gliv in morebiten antagonizem

Interakcije imajo zelo pomembno vlogo pri strukturnem oblikovanju glivnih združb (Falconer in sod., 2008). V naravnem okolju so interakcije izjemno kompleksne (Falconer in sod., 2008) in pomembno vplivajo na razporeditev, rastne vzorce in številčnost glivnih vrst (Yuen in sod., 1999; Badalyan in sod., 2004). Medvrstne interakcije lahko pomagajo razumeti spremembe v strukturi glivnih združb in načine uporabe gliv za biološko kontrolo patogenov (Badalyan in sod., 2004). Slednje je izjemno pomembno za zagotavljanje zdravih in stabilnih gozdov. Raziskave biološke kontrole bolezni lesnatih rastlin in dreves so redke v primerjavi s tistimi, ki jih izvajajo na zelnatih enoletnicah. Biološka kontrola sicer velja za obetavno alternativo naravni odpornosti rastlin in rabi kemičnih spojin za obvladovanje rastlinskih bolezni (He in sod., 2021). Po "širši" definiciji biološka kontrola obsega zmanjševanje oz. zatiranje škodljivih aktivnosti enega organizma z enim ali več organizmi, ki jih pogosto označujemo kot "naravne sovražnike". V okviru fitopatologije gre po navadi za uporabo antagonistov, ki prek različnih mehanizmov zavirajo ali celo ustavijo rast različnih patogenih gliv (Pal in McSpadden Gardener, 2006; Jenko, 2010). Pri tem velja opozoriti na zakonske določbe o uporabi domorodnih in tujerodnih vrst organizmov za namen biotičnega varstva. Za uporabo domorodnih vrst morajo biti izpolnjeni predpisani pogoji glede strokovnosti in tehnične usposobljenosti, pri uporabi tujerodnih vrst pa je postopek nekoliko bolj zapleten. Takšne vrste morajo biti namreč na seznamu, ki ga izda pristojno ministrstvo, in morajo pridobiti dovoljenje za vnos in uporabo Uprave Republike Slovenije za varno hrano, veterinarstvo in varstvo rastlin (Zakon o zdravstvenem varstvu rastlin, 2007).

Mehanizme antagonističnega delovanja v naravi lahko v prvi vrsti razdelimo na neposredne in posredne (Pal in McSpadden Gardener, 2006). V teoriji v nadaljevanju neposredne

mehanizme razdelimo na tekmovanje oz. kompeticijo, antibiozo in mikoparazitizem (Shearer in Zare-Maivan, 1988; Pal in McSpadden Gardener, 2006; Jenko, 2010). Glive po navadi tekmujejo za hranila in prostor. Ker je v določenih okoljih količina hranil omejena, nastane tekmovanje med njimi. Če je nekega hranila dovolj, se antagonistične značilnosti izničijo ali vsaj znatno zmanjšajo. Pri antibiozi nastajajo in se sproščajo hlapni in nehlapni sekundarni metaboliti oz. antagonistične spojine, ki zaviralno učinkujejo na vrste, ki so jim izpostavljene (Jenko, 2010; Prior in sod., 2017). V sklopu mikoparazitizma pa antagonistične glive oblikujejo različne encime (celulaze, hitinaze, glukanaze, proteinaze), ki delujejo neposredno na razkroj celičnih sten hif (Shearer in Zare-Maivan, 1988; Jenko, 2010). Tekmovanje in antibioza sta mehanizma, ki delujeta na daljavo, medtem ko je pri mikoparazitizmu značilen stik med patogenom in antagonistom (Pal in McSpadden Gardener, 2006). V okviru nekoliko težje razumljivega posrednega antagonizma pa govorimo o stimulaciji obrambnih mehanizmov in t. i. preobčutljivostnem odgovoru rastlin (Dangl in Jones, 2001). Z razlago slednjega bi krepko presegli začrtani okvir doktorske disertacije.

Pri pregledu literature smo našli številne raziskave na področju mikologije, ki se ukvarjajo z antagonističnimi učinki posameznih vrst. Hanácková in sod. (2017) so na primer proučevali antagonistične lastnosti endofitnih gliv na glivo *Hymenoscyphus fraxineus* v čistih kulturah. Badalyan in sod. (2004) so s parnimi primerjavami ugotavljali medsebojne vplive med nekaterimi trohnobnimi vrstami gliv in vrstami iz rodu *Trichoderma* in *Clonostachys*. Zasledili smo tudi nekaj raziskav konkurenčnosti odnosov med glivami prostotrosnicami, ki povzročajo razkroj lesa (Holmer in sod., 1997; Boddy, 2000). Wardle in sod. (1993) so raziskovali medsebojne vplive med glivami v naravnih substratih, tj. v tleh in gozdnem opadu. Raziskave antagonizma so relativno pogoste, vendar študij, ki bi se ukvarjale specifično z vrstami, ki se pojavljajo v odmrlih vejah gorskega javorja in njihovim vplivom na *E. parasitica,* ni.

1.1.4 Obarvanje in razkroj lesa

Les gorskega javorja in drugih vrst javorov je bele barve prek rumenkastobele do rahlo rdečkaste brez obarvane jedrovine. Lesa različnih vrst javorjev ne moremo zanesljivo razlikovati med seboj (Čufar, 2006). Traheje so v lesu razporejene difuzno oz. enakomerno s povprečnim premerom okoli 30 μm (Schumann in sod., 2019). Lahko so posamične ali v skupinah po 2 do 4 in imajo enostavne perforacije (Schoch in sod., 2004). Celične stene trahej so sestavljene iz srednje lamele (ML), primarne (P) in sekundarne (S) celične stene. Sekundarna stena se v nadaljevanju deli na tri sloje (S1, S2 in S3) glede na orientacijo mikrofibril. Celične stene v lesu so sestavljene iz osnovnih gradnikov oz. polimerov, tj. celuloze, hemiceluloze in lignina. V lesu je celuloze od 40 do 44 %, hemiceluloze 15 do 32 % in lignina 18 do 35 % (Goodell in sod., 2020). Celulozna vlakna dajejo lesu izjemno natezno trdnost. Lignin je zaradi raznolikosti povezovalnih vzorcev izjemno odporen proti

razkroju, ki ga "obvladajo" le določeni mikroorganizmi. Hemicelulozo pa lahko razumemo kot nekakšno povezavo med celulozo in ligninom, ki je najbolj občutljiva na razkroj med osnovnimi gradniki. Glive jo razkrojijo v začetnih fazah svojega delovanja (Goodell in sod., 2020). Celične stene trahej javorjev so na nekaterih mestih odebeljene (helikalne oz. spiralne odebelitve) (Čufar, 2006). Gostota trahej je od 150 do 160 n/mm^2 (Schumann in sod., 2019). Parenhimske celice so razpršene, apotrahealne (niso v stiku s trahejami) ali občasno paratrahealne (razporejene okoli trahej). Trakovi so večinoma 3- do 6-redni (Schoch in sod., 2004). Les javorjev je trd in srednje gost (povprečna gostota 590 kg/m^3), pri čemer je maklenov les nekoliko trši od lesa gorskega javorja. Les je homogen in podvržen okužbam z glivami in obarvanju oz. diskoloracijam (rjavo, zeleno, sivo, rumeno) (Čufar, 2006).

Okužbe z glivami lahko poleg obarvanja lesa povzročajo tudi njegov razkroj. Poznamo več tipov trohnobnih gliv, ki razkrojujejo les: glive povzročiteljice rjave trohnobe, bele trohnobe in mehke trohnobe. Glive povzročiteljice rjave ali destruktivne trohnobe imajo encime predvsem za razkroj celuloze in hemiceluloze. Nerazkrojen lignin daje lesu značilno temno barvo, največkrat rjavo. Les razpada na lističe in prizme, izgubi trdnost, je lahek ter se drobi v prah (Schwarze in sod., 2000; Maček, 2008; Goodell in sod., 2020). Glive, ki povzročajo belo ali korozivno trohnobo, z encimi razgrajujejo predvsem lignin, manj pa celuloze in hemiceluloze. Zato dobi les belkasto ali rumeno barvo. Pri tem trohnenje ni enakomerno, zato se v lesu pojavljajo elipsaste lise. Delovanje omenjenih gliv lahko povzroči tudi do 97 % izgubo mase (Schwarze in sod., 2000; Maček, 2008; Goodell in sod., 2020). Glive povzročiteljice mehke trohnobe imajo encime predvsem za razkroj celuloze in hemiceluloze, manj učinkovite pa so pri razkroju lignina. Kemijsko gledano je ta tip trohnobe bolj podoben rjavim trohnobam. Posebnost mehkih trohnob je rast hif v celicah lesa in oblikovanje votlinic okoli hif v sekundarni celični steni (S2). Za les je značilna spužvasta tekstura (Schwarze in sod., 2000; Schwarze, 2007). Večina gliv, ki povzročajo mehko trohnobo, spada med zaprtotrosnice in se pojavljajo v ekstremnejših, vlažnejših razmerah, ki ne ustrezajo tradicionalnim glivam rjave oz. bele trohnobe. Večinoma so te glive omejene na nekaj zunanjih milimetrov lesa in so zanimiva mešanica značilnosti rjavih in belih trohnob (Goodell in sod., 2020). Ločevanje tipov trohnob je do neke mere subjektiven proces, ki ga v zadnjem času dojemamo bolj kot kontinuiteto (Riley in sod., 2014). Zagotovo pa so definirane kategorije uporabne pri splošnem razvrščanju delovanja trohnobnih gliv (Goodell in sod., 2020).

Vsebnost vode v lesu posredno in neposredno vpliva na lastnosti lesa. Med neposrednimi so dimenzijske spremembe, med posrednimi pa vpliv vsebnosti vode na uspešnost naselitve in širjenja gliv v lesu (Goodell in sod., 2020). Najmanjša vsebnost vode v lesu za naselitev gliv je med 16 in 17 % (Zabel in sod., 2020). Vendar v večini primerov velja da mora biti vlažnost nad 25 %. Gre za t. i. točko nasičenja celičnih sten (angl. fiber saturation point), ko je vsa voda vezana v celične stene. Od 40 do 80 % vsebnosti vode je določen optimum za naselitev

gliv. Pri vsebnosti vode več kot 100 %, ko so z vodo nasičeni tudi lumni celic, pa se glive ne naseljujejo več na lesen substrat oz. vanj (Goodell in sod., 2020).

Z anatomskimi značilnostmi lesa in pravili razkroja je povezano tudi širjenje gliv v gostiteljevem lesu. Pri *E. parasitica* naj bi se hife po vstopu glive v gostitelja na začetku najhitreje razraščale vertikalno v trahejah, pozneje pa še v lateralni smeri prek pikenj v celičnih stenah (French, 1967). Hitro napredovanje glive v vertikalni smeri je povezano z eliptično obliko raka, ki se navadno razvije na površini okuženega debla. Traheje in trakovi so po raziskavi, ki jo je opravil French (1967), najbolj občutljivi za prisotnost in razrast glive. Tam je namreč z mikroskopsko analizo najpogosteje opazil tudi hife *E. parasitica*. Gliva naj bi prodirala skozi celične stene vlaken (osnovno tkivo z mehansko funkcijo) in trahej, deloma tudi parenhimskih celic trakov. Celoten mehanizem razraščanja gliv v lesu je povezan z osnovnimi zakonitostmi sistema CODIT (angl. compartmentalization of decay in trees) (Shigo in Marx, 1977). Kompartmentalizacija razkroja v drevesu je preživetveni mehanizem in eden izmed najpomembnejših odzivov drevesa na mehanske poškodbe ali okužbe. Model opisuje tvorbo štirih pregrad oz. sten, ki omejujejo nadaljnji razkroj lesa v drevesu. Stena 1 omejuje širjenje razkroja v vzdolžni smeri z aspiracijo pikenj ali odlaganjem depozitov, ki blokirajo prevodne elemente. Odvisno od hitrosti tega mehanizma nastanejo krajši ali daljši kompartmenti oz. predelki. Stena 2 omejuje širjenje razkroja v radialni smeri in jo predstavlja gostejši pozni les v vsaki braniki. Stena 3 omejuje širjenje razkroja v tangencialni smeri s pomočjo trakov, ki niso enakomerno razporejeni po celotnem drevesu, zato je tudi stena na nekaterih mestih prekinjena. Stena 4 je močnejša in bolj lokalizirana različica stene 2. Gre za barierno cono, ki jo tvori kambij in ločuje tkiva, nastala pred poškodbo in po njej. "Moč" posameznih sten se veča od prve proti četrti (Shigo in Marx, 1977).

Gliva *E. parasitica* povzroča obarvanje lesa in njegovo trohnenje. Razkroj lesne mase zaradi njenega delovanja na sladkornem, rdečem in srebrnem javorju je glede na podatke izredno počasna (French, 1967). Na splošno beležimo večje izgube mase v beljavi. Pri vseh treh vrstah javorja je po šestih mesecih izguba mase v jedrovini pri temperaturi 28 °C in konstantni vlažnosti znašala povprečno 1,29 %, v beljavi pa 2,47 % (French, 1967). Glede tipa trohnobe si raziskovalci niso enotni. Sinclair in sod. (1987) navaja, da *E. parasitica* povzroča počasno rjavo trohnobo, Worrall in sod. (1997) omenjajo mehko trohnobo, Pildain in sod. (2005) pa menijo, da vrste iz rodu Eutypella v začetku povzročajo mehko trohnobo, v nadaljevanju razkroja pa belo. Vsekakor ostaja vpliv *E. parasitica* na trohnenje naših najpogostejših domačih vrst javorjev (gorski javor, ostrolistni javor in maklen) neznan in ne raziskan. Okužbe z *E. parasitica* povzročajo veliko ekonomsko škodo, saj prizadenejo najvrednejši del debla (Kliejunas in Kuntz, 1974). Obolela drevesa imajo povprečno obarvanega in trohnečega 12 % skupnega volumna in 49 % prodajnega, kar pomeni polovično izgubo pri prodaji lesa na trgu (Gross, 1984b; Ogris in sod., 2005). Tudi Sendak in sod. (1997) so raziskovali vpliv *E. parasitica* na ekonomsko škodo in izračunali 0,33

m^3/ha izgube skupnega volumna pri sladkornem javorju, kar so ugotovili s pomočjo vizualno ocenjenega razreda razkroja lesa. Nekaj raziskav razkroja lesa z *Eutypella* sp. so izvajali tudi na drugih drevesnih vrstah. Pildain in sod. (2005) so na primer proučevali razkroj lesa ameriškega črnega topola (*Populus deltoides* W. Bartram ex Marshall) v 12-tedenskem poskusu s štirimi izolati vrst iz rodu *Eutypella*. Povprečna izguba mase je znašala 17,6 %. Worrall in sod. (1997) pa so proučevali razkroj beljave rumene breze (*Betula alleghaniensis* Britto) in bora (*Pinus taeda* L.). V poskus so vključili tudi izolate *E. parasitica* in po dvanajsti tednih ugotovili 5,6 % izgubo mase pri brezi in 2,3 % izgubo pri boru.

Les javorjev dosega visoke cene. Že nekaj let zapored je gorski javor najbolje ocenjen na dražbi vrednejšega lesa v Slovenj Gradcu (Katalog sortimentov, 2017, 2019, 2020). Včasih ima namreč les zaradi različnih zunanjih vplivov posebno teksturo (javor ikraš, javor rebraš in ptičji javor), zaradi česar je še posebno dragocen (Brus, 2008). Tudi s tega vidika je dobro večjo pozornost nameniti raziskavam na področju razvrednotenja zaradi delovanja *E. parasitica*.

Gliva *E. parasitica* je relativno slabo raziskana vrsta. Poleg osnovnih starejših severnoameriških študij (Davidson in Lorenz, 1938; French, 1967; French, 1969; Lachance in Kuntz, 1970; Lachance, 1971; Kliejunas in Kuntz, 1972, 1974; Johnson in Kuntz, 1976, 1979; Glawe, 1983; Gross, 1984a, 1984b; Sendak in sod., 1997) in poznejših poročil o novih najdbah iz evropskih držav (Cech, 2007; Ogris in sod., 2008; Cech in sod., 2016; Jurc in sod., 2016; Černý in sod., 2017; Bregant, 2018; Jurc in sod., 2019; Dubach in sod., 2022) ni novejših člankov, ki bi obravnavali biološke in ekološke značilnosti omenjene vrste. Pomanjkanje raziskav in želja po novih odkritjih sta bila glavna povoda za nastanek tega dela.

1.2 PREDSTAVITEV NAMENA, CILJEV IN HIPOTEZ

Naš glavni namen je bil raziskava izbranih področij biologije vrste *E. parasitica*, ki povzroča javorov rak, njenega vpliva na izgubo mase lesa treh vrst javorjev in odnosov z drugimi najpogostejšimi vrstami gliv, ki se pojavljajo v odmrlih vejah gorskega javorja. Z raziskavo smo skušali pridobiti razširjeno znanje o vrstah gliv in raziskati njihov vpliv na uspešnost naselitve in razrasti *E. parasitica* v odmrlih vejah gorskega javorja. Z raziskavo smo želeli odkriti in predlagati nove, biološke načine za zatiranje javorovega raka, kar bi bilo lahko zanimivo in uporabno ne le za Slovenijo, temveč tudi za širše območje Evrope in Severne Amerike, kjer se bolezen pojavlja. Ker *E. parasitica* navadno poškoduje spodnji in s tem tudi najdebelejši najvrednejši sortiment, smo želeli z rezultati raziskave o izgubi mase lesa pripomoči tudi k boljšemu razumevanju vpliva glive na ekonomsko in tehnično razvrednotenje okuženih javorjev.

V okviru doktorske disertacije smo v ta namen postavili raziskovalne cilje.

Cilj 1: Določiti najpogostejše vrste gliv v lesu odmrlih vej gorskega javorja na petih lokacijah v okolici Ljubljane.

Cilj 2: Izmeriti vpliv desetih najpogostejših vrst gliv v lesu odmrlih vej gorskega javorja na rast glive *E. parasitica* v čisti kulturi in ugotoviti njihov morebitni antagonizem.

Cilj 3: Ugotoviti izgubo lesne mase treh vrst javorjev (*Acer pseudoplatanus*, *A. platanoides*, *A. campestre*) zaradi delovanja glive *E. parasitica* po metodi mini-blok, ki temelji na standardu SIST EN 113:2002, in primerjati rezultate z izolati gliv *Trametes versicolor*, *Gloeophyllum trabeum* in s petimi najpogostejšimi glivami, ki povzročajo trohnobo v javorjevih odmrlih vejah.

Znotraj posameznih ciljev smo postavili tudi raziskovalne hipoteze.

Hipoteza 1: Glivne združbe v lesu odmrlih vej gorskega javorja (*A. pseudoplatanus*) se značilno razlikujejo med različnimi lokacijami vzorčenja v osrednji Sloveniji (Rožnik, Smrekovec, Mokrc, Mala voda, Samotorica), med različnima mestoma izolacije (veja, deblo) in med različnimi debelinskimi razredi vej (od 0,2 do 0,7 cm, od 0,8 do 1,2 cm, od 1,3 do 3,2 cm).

Hipoteza 2: Vrednost Shannonovega diverzitetnega indeksa vrst gliv izoliranih iz lesa odmrlih vej gorskega javorja (*A. pseudoplatanus*) med različnimi lokacijami vzorčenja v osrednji Sloveniji (Rožnik, Smrekovec, Mokrc, Mala voda, Samotorica), med različnima mestoma izolacije (veja, deblo) in med različnimi debelinskimi razredi vej (od 0,2 do 0,7 cm, od 0,8 do 1,2 cm, od 1,3 do 3,2 cm) je višja od 3.

Hipoteza 3: Gliva *E. parasitica* je v čisti kulturi šibko konkurenčna desetim najpogosteje izoliranim vrstam gliv v lesu odmrlih vej gorskega javorja (*A. pseudoplatanus*).

Hipoteza 4: Gliva *E. parasitica* povzroči manjše izgube lesne mase gorskega javorja (*A. pseudoplatanus*), ostrolistnega javorja (*A. platanoides*) in maklena (*A. campestre*) v primerjavi z glivama razkrojevalkama *Trametes versicolor* in *Gloeophyllum trabeum*.

2 ZNANSTVENA DELA

2.1 *Eutypella parasitica* IN DRUGE POGOSTO IZOLIRANE VRSTE GLIV IZ LESA ODMRLIH VEJ MLADIH GORSKIH JAVORJEV (*Acer pseudoplatanus*) V SLOVENIJI

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Eutypella parasitica R. W. Davidson and R. C. Lorenz je povzročiteljica javorovega raka, uničujoče bolezni javorjev v Evropi in Severni Ameriki. Gliva *E. parasitica* okuži gostiteljevo deblo prek odmrlih vej ali ran v skorji. Zaradi morebitnega vpliva glivne združbe na okužbo in razrast *E. parasitica* smo na petih vzorčnih lokacijah v Sloveniji proučili sestavo gliv v lesu odmrlih vej gorskega javorja (*Acer pseudoplatanus* L.). Od novembra 2017 do marca 2018 smo na vsaki vzorčni lokaciji nabrali 40 vzorcev vej. Izolacije smo izvedli iz lesa v zunanjem delu odmrle veje in iz razbarvanega lesa v deblu, ki je izviral iz odmrle veje. Čiste kulture smo razvrstili v morfotipe in izbrali po eno reprezentativno kulturo za morfotip za nadaljnjo molekularno določitev. Iz skupno 2700 nacepljenih koščkov se je razvilo 1744 glivnih kultur, ki smo jih razvrstili v 212 morfotipov. V preiskovanih vzorcih so bile najpogosteje izolirane vrste *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 in *E. parasitica*. V raziskavi smo ločevali med vrstno pestrostjo in združbo gliv. Razlike v pestrosti glivnih vrst med petimi vzorčnimi lokacijami niso bile statistično značilne, prav tako se tudi debelina vej ni izkazala za statistično značilen dejavnik vrstne pestrosti gliv. Kljub temu so relativno nizke vrednosti Jaccardovega indeksa podobnosti kazale na morebitne razlike v združbi gliv med različnimi vzorčnimi lokacijami. Analiza podobnosti je pokazala, da se izolirane združbe gliv statistično značilno razlikujejo med petimi vzorčnimi lokacijami in med različnimi mesti izolacije. *Eutypella parasitica* smo izolirali iz vseh petih vzorčnih lokacij, čeprav smo javorove rake opazili le na treh lokacijah, kar kaže na možnost asimptomatskih okužb.

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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., ***Trichoderma spp.* Pers.**
Johnova [31] [4]	decayed trunks, stumps, twigs and branches	***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

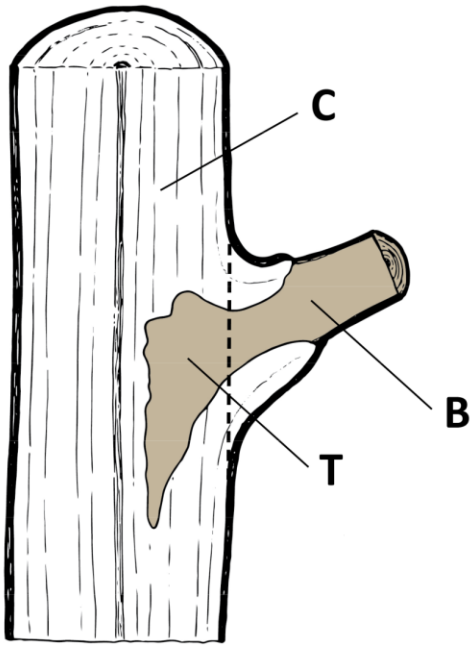

Figure 1. Sources of fungal isolations in a sample: B, branch; T, trunk; C, control (illustration by S. Zidar, Slovenian Forestry Institute).

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	1.41	1.83	809
	MN240814			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	2.75	1.79	813
	MN244538			812
	MN240810			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	1.28	2.50	814
	MN244541			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	2.05	2.46	818
	MN244550			819
	MN244548			816
Diaporthe sp. 2	MN240816	2.18	2.00	788
Epicoccum nigrum Link	MN216311	2.37	2.31	820
	MN244547			817
Eutypa lata (Pers.) Tul. and C. Tul.	MN252417	2.69	3.50	821
	MN252418			822
	MN252420			824
Eutypa maura (Fr.) Sacc.	MN252421	8.77	2.91	789
	MN252423			826
Eutypa spp. Tul. and C. Tul.		11.77	2.83	
Eutypa sp. 1	MN252415	2.24	2.69	828
	MN252411			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	1.92	3.00	845
	MN976066			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).

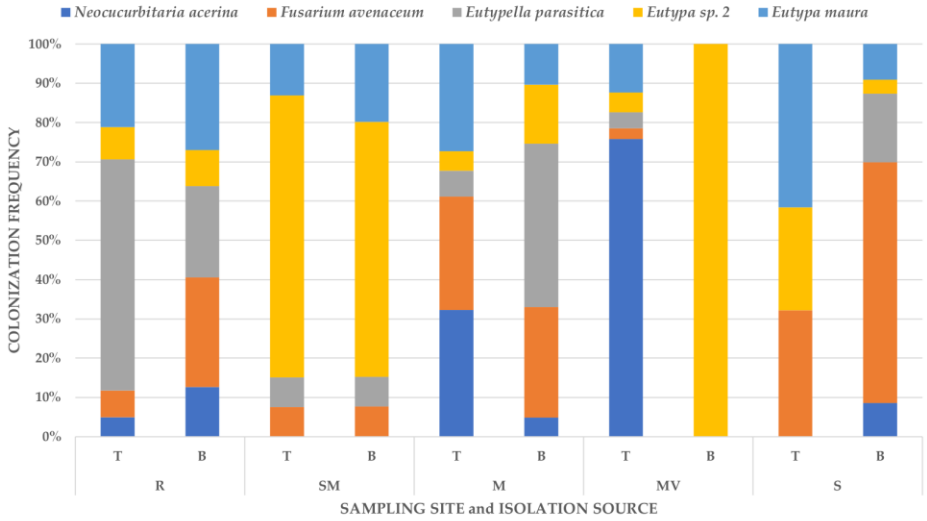

Figure 2. Colonization frequency of the five most frequently isolated fungi of wood of dead branches of young *A. pseudoplatanus* from different sampling sites and isolation sources. Sampling site: R, Rožnik; SM, Smrekovec; M, Mokrc; MV, Mala voda; S, Samotorica; Isolation source: T, discoloured wood 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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2.2 *In vitro* INTERAKCIJE MED *Eutypella parasitica* IN NEKATERIMI POGOSTO IZOLIRANIMI GLIVAMI IZ LESA ODMRLIH VEJ GORSKEGA JAVORJA (*Acer pseudoplatanus*)

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Za oceno *in vitro* antagonistične aktivnosti desetih najpogosteje izoliranih gliv iz lesa odmrlih vej *Acer pseudoplatanus* L. na *Eutypella parasitica* R. W. Davidson and R. C. Lorenz, povzročiteljico uničujoče bolezni javorjev v Evropi in Severni Ameriki, smo naredili teste dvojnih kultur. Testirali smo t. i. izzivalne izolate gliv *Diaporthe* sp., *Eutypa* sp., *Eu. maura*, *E. parasitica*, *Fusarium avenaceum*, *Neocucurbitaria acerina*, *Neonectria* sp., *Peniophora incarnata*, *Petrakia irregularis* in *Phomopsis pustulata*. Antagonistični potencial posameznega izolata smo ocenili z izračunom antagonističnega indeksa (*AI*), ki temelji na tipu interakcije v dvojnih kulturah. Rezultat tekmovanja med glivnimi izolati smo opredelili po reizolacijah iz interakcijske cone (*s*). V dvojnih kulturah sta bila opazna dva glavna tipa interakcij: zaustavitev rasti kot posledica medsebojnega zaviranja po micelijskem stiku ali brez njega, in zamenjava, ki se odraža v zaviranju rasti *E. parasitica*, čemur sledi delno preraščanje z micelijem izzivalnega izolata. S statistično analizo smo ugotovili statistično značilne razlike v povprečni vrednosti *AI* in *s* med izzivalnimi izolati v dvojnih kulturah. Na podlagi rezultatov antagonističnega indeksa so imele *Eutypa* sp., *Eu. maura*, *Neonectria* sp. in *P. incarnata* največji zaviralni učinek na rast *E. parasitica* in smo jih prepoznali kot najboljše kandidate za nadaljnje študije o biološki kontroli *E. parasitica*. Micelij *E. parasitica* v interakcijski coni je večinoma ostal aktiven, razen v dvojnih kulturah z *Eutypa* sp., *F. avenaceum* in *Neonectria* sp., kjer reizolacije *E. parasitica* niso bile uspešne. Na podlagi rezultatov predvidevamo, da je *E. parasitica* šibek tekmec, ki vlaga manj energije v neposredno micelijsko tekmovanje. Razpravljamo o potencialu opazovanih antagonistov kot možne biološke kontrole javorovega raka. Kljub vsemu so potrebni dodatni poskusi za trdne zaključke o konkurenčni sposobnosti *E. parasitica* in uporabnosti antagonistov za biološko kontrolo.

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In Vitro Interactions between *Eutypella parasitica* and Some Frequently Isolated Fungi from the Wood of the Dead Branches of Young Sycamore Maple (*Acer pseudoplatanus*)

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Abstract: The ten most frequently isolated fungi from the wood of the dead branches of *Acer pseudoplatanus* L. were tested in dual cultures to evaluate their in vitro antagonistic activity against *Eutypella parasitica* R.W. Davidson and R.C. Lorenz, the causative agent of a destructive disease of maples in Europe and North America. The tested fungi, treated also as challenge isolates, were *Diaporthe* sp., *Eutypa* sp., *Eu. maura*, *E. parasitica*, *Fusarium avenaceum*, *Neocucurbitaria acerina*, *Neonectria* sp., *Peniophora incarnata*, *Petrakia irregularis*, and *Phomopsis pustulata*. The antagonistic ability of each challenge isolate was evaluated by calculating an index of antagonism (*AI*) based on the interaction type in the dual cultures. The results of competition between the fungal isolates were quantified after re-isolations from the interaction zone (*s*). The dual cultures revealed two main types of competitive interactions: Deadlock, consisting of mutual inhibition after mycelial contact or at a distance, and replacement, reflecting in the inhibition of *E. parasitica*, followed by partial overgrowth by the replacing fungus. Statistical analysis showed significant differences in average *AI* and *s* of challenge isolates between different dual culture assays. Based on the results of the antagonism index, *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* had the highest inhibitory effect on *E. parasitica* growth and were recognized as the most promising candidates for further biocontrol studies of *E. parasitica*. The mycelium of *E. parasitica* at the interaction zones remained mostly viable, except in dual cultures with *Eutypa* sp., *F. avenaceum*, and *Neonectria* sp., where re-isolations did not yield any colony of the *E. parasitica* isolate. Based on the results, we assume that *E. parasitica* is a weak competitor, which invests less energy in direct mycelial competition. We discuss the potential of the observed antagonists as a possible biocontrol of Eutypella canker of maple. Nevertheless, additional experiments should be performed for a solid conclusion about competitive ability of *E. parasitica* and usefulness of antagonists as biocontrol.

Keywords: *Eutypella parasitica*; dual culture; hyphal interaction; deadlock; replacement; competition; antagonism; inhibition; re-isolation; biocontrol

1. Introduction

Interactions play a significant role in shaping the community structure of fungal organisms [1]. They are an important determinant of the distribution, growth pattern, and abundance of fungal species in any natural fungal community [2,3] and can also be used for developing biocontrol strategies. Interactions in the natural environment are complex [1] and have been studied using a variety of

techniques, including observations of hyphal interactions, tests of inhibition on hyphal growth, and examination of reaction types [3]. Some fungal endophytes are known for their defensive power against various tree pathogens [4], but there are also concerns about fungi having the opposite function of contributing to the development of tree diseases [5–7]. Endophytes can influence the presence of pathogens in a tree by different mechanisms, i.e., antibiosis by metabolites, competition for nutrients or space, mycoparasitism, and indirect effects including induced systemic resistance of the tree [8,9].

Interactions between fungal mycelia commonly result in distinctive changes along interaction zones [10]. The outcome of interspecific fungal interactions depends on species compatibility [11], as well as the microclimate and physio-chemical structure of the substrate [12]. The fungal interaction outcome is a complex phenomenon and may be one of the following: (1) Deadlock, i.e., neither isolate enters the territory of the other, or (2) replacement, i.e., one isolate is partially or entirely replaced by the other [13]. Deadlock usually occurs due to each isolate excreting and/or detecting "non-native" chemical compounds that inhibit growth [14]. This type of interaction commonly occurs following, but sometimes also prior to, mycelial contact [15]. Partial replacement occurs when one fungus initially gains headway but subsequently stops, or when both species make some entry into the territory of the other [12]. In contrast, complete replacement results from one individual fungus completely engulfing the other. In addition to deadlock and replacement, Boddy [12] also used a third possible outcome of an interaction—intermingling, i.e., a neutral interaction that results in the fusion of colonies for compatible genotypes or spatial intermixing for incompatible genotypes.

The fungus *Eutypella parasitica* R.W. Davidson and R.C. Lorenz, the causative agent of Eutypella canker of maple, was reported for the first time in Europe from Slovenia [16]. Later, the disease was also reported from other regions in Europe [17–23]. It is believed to originate from North America [24] and to represent a considerable risk for naturally distributed maples in Europe [22,25]. The fungus *E. parasitica* most likely enters the trunk through branch stubs or bark wounds [26] where it competes with other fungal species. The fungal species *Eutypa* sp. Tul. and C. Tul., *Eutypa maura* (Fr.) Sacc., *Fusarium avenaceum* (Fr.) Sacc., *Neocucurbitaria acerina* Wanas., Camporesi, E.B.G. Jones and K.D. Hyde, *Diaporthe* sp. Nitschke, *E. parasitica*, *Neonectria* sp. Wollenw., *Petrakia irregularis* Aa, *Phomopsis pustulata* (Sacc.) Died., and *Peniophora incarnata* (Pers.) P. Karst. are some of the most frequently isolated species from the wood of the dead branches of young sycamore maple [27]. However, little is known about how they interact and compete for the same substrate.

The aim of the present study was to investigate the in vitro activity of the ten most frequently isolated fungal species from the wood of the dead branches of *Acer pseudoplatanus* L. [27] against *E. parasitica* in dual culture experiments.

2. Materials and Methods

2.1. Interactions between E. parasitica and Ten Fungal Isolates in Dual Cultures

The dual culture technique was used to test the possible antagonistic effect of ten fungal isolates isolated from the wood of the dead branches of *A. pseudoplatanus* [27] against an isolate of *E. parasitica* (ZLVG 805, see Table 1). The reference isolate of *E. parasitica* was obtained from Eutypella canker of maple (46.0533° N, 14.4914° E, 338 m a.s.l.) on *A. pseudoplatanus* and is referred to as the "response isolate" throughout the paper. Other fungal isolates used in the experiment are referred to as "challenge isolates", including one additional isolate of *E. parasitica* (ZLVG 791).

Interactions between isolates were performed in plastic Petri dishes (Ø = 90, *h* = 15 mm) containing 3.9% (w/v) potato dextrose agar (PDA; Becton Dickinson, Sparks, Maryland, United States). Agar discs (Ø = 5 mm) taken from the margin of actively growing, one-week-old cultures were placed 4 cm apart from each other on the PDA. The cultures were incubated at 24 °C in the dark (I-190 CK incubator; Kambič, Semič, Slovenia).

For the self-inhibition test, two discs of *E. parasitica* (ZLVG 805) taken from the same colony were used. The control consisted of pairing *E. parasitica* (ZLVG 805) with a sterile agar disc. Each combination

was replicated three times, following examples in the literature [4,28–30]. A total of 36 interactions were examined, including the self-inhibition assays and control pairings.

Table 1. Fungal isolates [1] used in dual cultures assays.

Fungal Isolate	Collection Number [2]	Number of Days [3]
Diaporthe sp. Nitschke	ZLVG 788	12
Eutypa sp. Tul. & C. Tul.	ZLVG 790	7
Eutypa maura (Fr.) Sacc.	ZLVG 789	5
Eutypella parasitica R.W. Davidson & R.C. Lorenz	ZLVG 791	12
Eutypella parasitica R.W. Davidson & R.C. Lorenz [4]	ZLVG 805	6
Fusarium avenaceum (Fr.) Sacc.	ZLVG 792	12
Neocucurbitaria acerina Wanas., Camporesi, E.B.G. Jones & K.D. Hyde	ZLVG 794	—
Neonectria sp. Wollenw.	ZLVG 795	6
Peniophora incarnata (Pers.) P. Karst.	ZLVG 797	5
Petrakia irregularis Aa	ZLVG 798	14
Phomopsis pustulata (Sacc.) Died.	ZLVG 799	7

[1] The ten most frequently isolated fungi from the wood of the dead branches of *A. pseudoplatanus* [27] as challenge isolates and the response isolate of *E. parasitica*. [2] ZLVG—Culture collection of the Laboratory of Forest Protection at the Slovenian Forestry Institute. [3] Number of days required for each fungal isolate to form a two-centimeter-diameter colony at 24 °C in the dark, also referred to as growth rate. [4] Response isolate of *E. parasitica* paired with the ten challenge fungal isolates.

Based on differences in the growth rate of each fungal isolate in comparison to the response isolate of *E. parasitica*, the isolates were inoculated into Petri dishes on different starting days to give them a chance to meet in the center of the plate (Table 1). We therefore carried out a preliminary test of the growth rate of the fungal isolates. Agar discs (Ø = 5 mm) taken from the margin of actively growing cultures were cut and placed in the center of 90-mm diameter plastic Petri dishes containing 3.9% PDA and incubated at 24 °C in the dark (I-190 CK incubator; Kambič, Semič, Slovenia). We counted the number of days required for each fungal isolate to form a two-centimeter-diameter colony (Table 1). Slower growers were placed on the agar in advance of *E. parasitica*, while faster growers were placed on the agar after *E. parasitica*.

The antagonistic ability of each fungal isolate against *E. parasitica* was examined daily and scored using the Badalyan, Innocenti, and Garibyan [13] rating for a macroscopically determined type of interaction. A rating scale for the three main and four sub-types of interactions was used (Table 2).

Table 2. Types and subtypes of fungal interactions with corresponding scores [13].

Label	Type of Interaction	Score
A	Deadlock [1] at mycelial contact	1
B	Deadlock at a distance, without mycelial contact	2
C	Replacement [2]	3
C_{A1}	Partial replacement after initial deadlock with mycelial contact	3.5
C_{B1}	Partial replacement after initial deadlock at a distance	4
C_{A2}	Complete replacement after initial deadlock with mycelial contact	4.5
C_{B2}	Complete replacement after initial deadlock at a distance	5

[1] Mutual inhibition in which neither isolate was able to overgrow the other. [2] Overgrowth without initial deadlock.

An antagonism index (*AI*) [13] was calculated for each challenge fungal isolate using the equation:

$$AI = \sum n \times i, \quad (1)$$

where n is the number of each type or sub-type of interaction in dual cultures and i is the corresponding score (Table 2). *AI* is a qualitative measure defined as the ability of a fungus to dominate and

compete with other species [31]. Higher *AI* denotes higher competitive and inhibitory ability of a challenge isolate.

2.2. Re-Isolations from Dual Cultures

Ten days after mycelium contact or ten days after no mycelium growth on the connective line between both colonies, the observed results of the competition were quantified after fungal re-isolation. Five agar discs (Ø = 5 mm) were cut from the interaction zone or from the margin of the *E. parasitica* colony (in the case of no mycelium contact), and two agar discs (one per isolate) were cut from areas with a presumed growth of only one individual fungus (Figure 1) [32]. To confirm the viability of *E. parasitica* in co-cultures, discs were placed on 3.9% (*w/v*) PDA plates and incubated at 24 °C in the dark (I-190 CK incubator; Kambič, Semič, Slovenia). The outgrown mycelium cultures were compared to the original isolates, and the identity of *E. parasitica* cultures was additionally confirmed by using *E. parasitica* specific primers (EpF/R). The methodology followed that described by Piškur, et al. [33], except for DNA extraction, which was done 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). Reactions that yielded a single and strong fragment size of 341 bp on the electrophoresis gel were classified as *E. parasitica* positive [33].

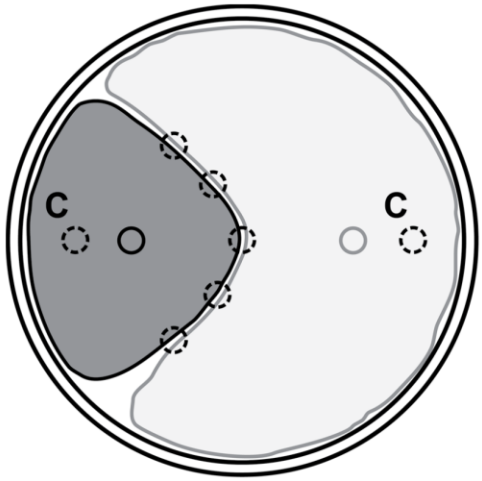

Figure 1. Design of the re-isolation test from a Petri dish with competing mycelia: Five discs taken from the interaction zone and two control discs (C) from the colony margins. The gray culture is the response isolate of *E. parasitica*, and the white culture is the challenge isolate (illustration by S. Zidar, Slovenian Forestry Institute).

The number of successful re-isolations per individual isolate in each interaction was counted. The re-isolation success of isolate A (challenge isolate) in a dual culture assay with isolate B (*E. parasitica*) was quantified as the sum of re-isolated fungal isolates from each Petri dish. These sums were log-transformed according to Koukol, Mrnka, Kulhankova, and Vosatka [32]:

$$s = \ln((A+1)/(B+1)), \quad (2)$$

where s is the score quantifying the re-isolation success of a challenge isolate in the interaction, and A and B are the mean number of successfully re-isolated challenge and response fungal isolates, respectively (n = max 5). The estimated value of s ranges between −1.79 and +1.79. Higher values of s indicate higher average re-isolation success, while lower values of s indicate lower average re-isolation success of a challenge isolate in competition with *E. parasitica*.

A non-parametric Kruskal–Wallis test was used to compare average antagonistic ability and average re-isolation success of a challenge isolate between different dual culture assays. Afterwards, a post-hoc multiple comparison Dunn test with Benjamini–Hochberg adjustment was used. To test the assumption of normality, the Shapiro–Wilk's test was applied, and for testing the homogeneity of variances, the Levene's test was used.

All calculations and graph design were performed in Microsoft Excel version 16.0.12527.20612. Statistical analyses were performed in the R software environment for statistical computing [34] with the "car" [35], "FSA" [36], and "rcompanion" packages [37]. Mycelial interactions were photographed using a Panasonic Lumix DMC-FZ7 digital camera (Panasonic, Osaka, Japan).

3. Results

3.1. Interactions between E. parasitica and Ten Fungal Isolates in Dual Cultures

Macroscopic examination of dual cultures revealed that almost all challenge isolates made hyphal contact with the response isolate, i.e., *E. parasitica*, within the time of the experiment. *Peniophora incarnata* was the most aggressive challenge isolate, almost completely replacing *E. parasitica* (Figure 2g). Moreover, we did not observe any macroscopic changes in the mycelium of *E. parasitica* in any of the tested dual cultures. In co-cultures of *F. avenaceum*, *N. acerina*, *Pe. irregularis*, and *Ph. pustulata*, a large quantity of spores around the agar disc of the response isolate was observed.

Dual culture assays showed a diverse pattern of interaction types between the response and challenge fungal isolates (Table 3). In four assays we found an interaction in which *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* partially replaced *E. parasitica* after initial deadlock with mycelium contact (interaction type C_{A1}) (Figure 2b,c,f,g). In five other assays, the challenge and the response isolate inhibited each other's growth after initial mycelial contact and neither isolate was able to overgrow the other (interaction type A) (Figure 2a,d,e,h,i). In just two cases (*Diaporthe* sp. and *N. acerina*), we observed deadlock at a distance, without mycelial contact (interaction type B), but since this was not the predominant type of the specific assay, we did not use it as a representative type of interaction. However, we did not observe any case in which *E. parasitica* overgrew the challenge isolate. The types of interactions were reflected in the calculated antagonism index (*AI*) (Table 3). Most of the challenge isolates achieved low *AI* values. Of the challenge isolates, *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* had the strongest antagonistic effect (Table 3).

Table 3. Interaction type and antagonism index (*AI*) of *E. parasitica* in dual culture assays.

Fungal Isolate	Interaction Type [2]	*AI*	Statistic Group [4]
Diaporthe sp.	A [3]	4	ab
Eutypa sp.	C_{A1}	10.5	a
Eutypa maura	C_{A1}	10.5	a
Eutypella parasitica (ZLVG 791)	A	3	b
Eutypella parasitica (ZLVG 805) [1]	A	3	b
Fusarium avenaceum	A	3	b
Neocucurbitaria acerina	A [3]	4	ab
Neonectria sp.	C_{A1}	10.5	a
Peniophora incarnata	C_{A1}	10.5	a
Petrakia irregularis	A	3	b
Phomopsis pustulata	A	3	b

[1] Self-inhibition assay. [2] Type of interaction followed the classification of Badalyan, Innocenti, and Garibyan [13], see Table 2. [3] In one out of three Petri dishes, the B interaction type was determined. [4] Different letters indicate significant differences ($p < 0.05$).

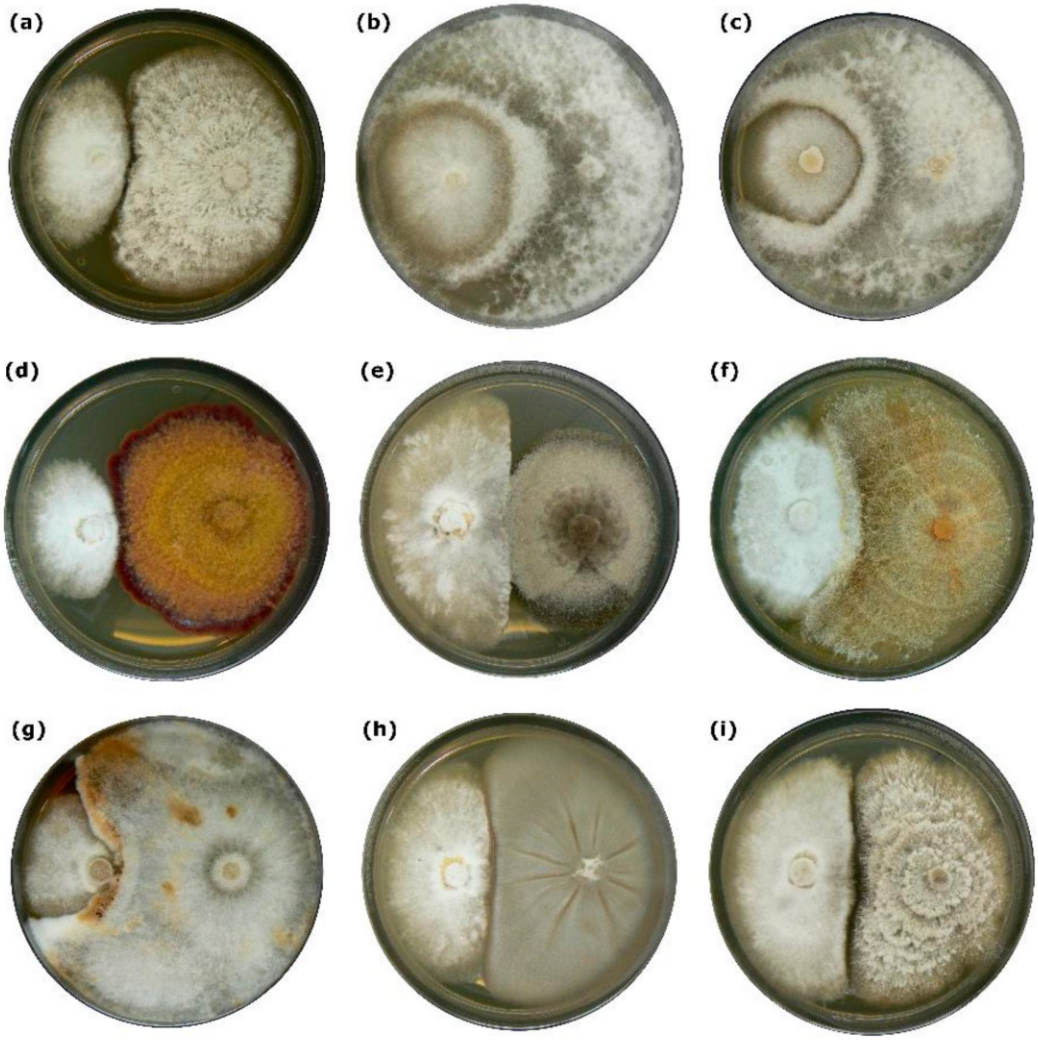

Figure 2. Mycelial interactions after 18 days of co-incubation between the response isolate of *E. parasitica* (left) and the challenge isolate (right): (**a**) *Diaporthe* sp.; (**b**) *Eutypa* sp.; (**c**) *Eu. maura*; (**d**) *F. avenaceum*; (**e**) *N. acerina*; (**f**) *Neonectria* sp.; (**g**) *P. incarnata*; (**h**) *Pe. irregularis*; and (**i**) *Ph. pustulata*. Note: In other replicates different types of interactions could be observed (Table 3).

In co-cultures with *Eutypa* sp., *Eu. maura*, *Neonectria* sp., *P. incarnata*, *Pe. irregularis*, and *Ph. pustulata*, the formation of a distinctive interaction zone could be observed (Figure 2b,c,f–i). Contact of fungal isolates with each other resulted in yellowish brown, sometimes even black, pigmentation and formation of dense mycelium. Dense mycelium at the interaction zone of interaction type A was usually produced by both isolates (Figure 2e), while in interaction type C_{A1}, dense aerial mycelium, was produced only by the overgrowing isolate (Figure 2b,c,f,g). Changes in pigmentation of the interaction zone were best observed from the reverse side of the Petri dishes.

In a self-inhibition test and dual culture assay with a different isolate of *E. parasitica*, colonies almost uniformly showed interaction type A (Figure 3), where neither isolate was able to overgrow the other. From six replicated co-cultures (three for self-inhibition test and three for dual culture assay), a dense mycelium at the interaction zone was formed only in one case. For the control pairings of *E. parasitica* and sterile agar discs there was no reason to determine the interaction type.

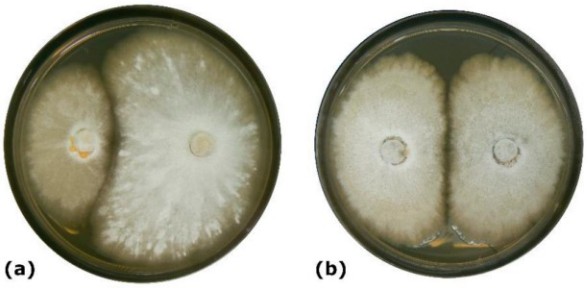

Figure 3. Mycelial interactions after 18 days of co-incubation between: (**a**) Response (left) and challenge (right) isolate of *E. parasitica* and (**b**) two response isolates of *E. parasitica* (self-inhibition).

The results of the Kruskal–Wallis statistical test revealed significant differences ($p < 0.001$) in the average antagonistic ability of challenge isolates between different dual culture assays. Pairwise comparisons revealed statistically significant differences ($p < 0.05$) for the average *AI* score between *E. parasitica* ZLVG 791 (a dual culture assay) on one hand and *Eutypa* sp., *Eu. maura*, *Neonectria* sp. and *P. incarnata* on the other. The same results were obtained with *E. parasitica* ZLVG 805 (a self-inhibition test) (Table 3).

3.2. Re-Isolations from Dual Cultures

Out of 189 re-isolated discs, 2.1% did not develop any colony, while in 24.3% of cases both challenge and response isolates were re-isolated from the interaction zone. No re-isolations of *E. parasitica* were yielded after interaction with *Eutypa* sp., *F. avenaceum*, and *Neonectria* sp., which were the most successful isolates regarding the number of successful re-isolations. In contrast, *Diaporthe* sp., *N. acerina*, and *Pe. irregularis* were re-isolated less successfully (Figure 4).

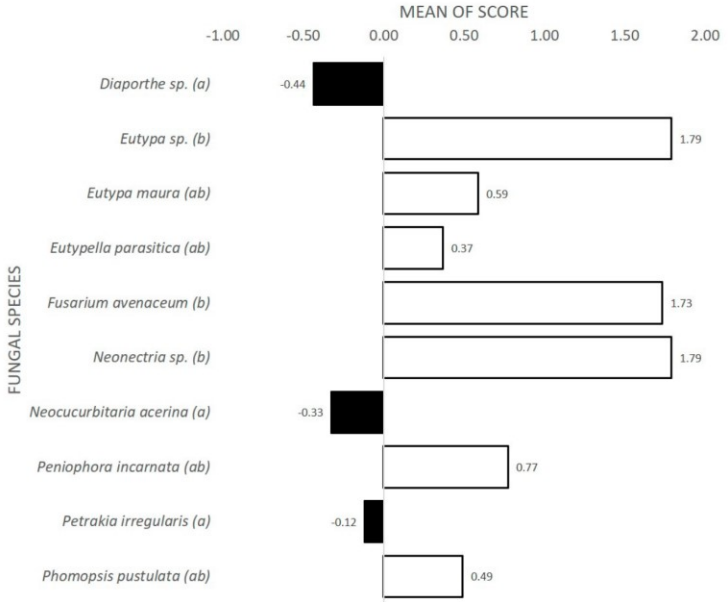

Figure 4. Mean scores (*s*, Equation (2), [−1.79, +1.79]) of re-isolation of fungal isolates from dual cultures quantifying the re-isolation success of a challenge isolate in the interaction zone. Bars above zero refer to higher average re-isolation success, while bars below zero refer to lower average re-isolation success of the challenge isolate in competition with *E. parasitica*. Different letters in parenthesis, indicate significant differences ($p < 0.05$).

Seven out of ten challenge isolates showed positive re-isolation success (Figure 4). For *E. parasitica* in the self-inhibition test, the average value of *s* was determined at 0.00 because we could not differentiate among the same isolate after re-isolation. Similarly, for the control pairings of *E. parasitica* and sterile agar discs, we did not obtain the average value of *s* because there was no challenge isolate which could influence the growth of *E. parasitica*.

The results of the Kruskal–Wallis statistical test revealed significant differences ($p < 0.01$) in the average re-isolation success of challenge isolates between different dual culture assays. Pairwise comparisons revealed statistically significant differences ($p < 0.05$) for the average re-isolation score between *Eutypa* sp., *F. avenaceum*, and *Neonectria* sp. on one hand and *Diaporthe* sp., *N. acerina*, and *Pe. irregularis* on the other. No statistically significant differences ($p < 0.05$) were obtained for any of the pairwise comparisons with *E. parasitica*.

4. Discussion

4.1. Interactions between E. parasitica and Ten Fungal Isolates in Dual Cultures

Based on the *AI* values, fungal isolates can be divided into three categories according to Badalyan, Innocenti, and Garibyan [13]: (1) Active, with $AI > 15$; (2) moderately active, with *AI* between 10 and 15; and (3) weakly active, with $AI < 10$. A lower index of antagonism indicated that the challenge isolate was weaker in terms of its inhibition of the response isolate [3]. Our experiment revealed mostly weakly active challenge isolates. Only *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* were moderately active in inhibition according to the index of antagonism.

Because of the occasionally very similar macroscopic appearance of co-incubated mycelia, it was difficult to determine the type of interaction or to select the isolate which overgrew the other. It was especially difficult to differentiate between interaction type A and B. However, *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* were the only challenge isolates that overgrew the response isolate (interaction type C_{A1}). This result suggests that these isolates have the greatest potential as biocontrol agents, based on the *AI* values. Consistent with the general results of other research on fungal interactions [30], two types of interactions were observed.

Eutypella parasitica did not overgrow any of the challenge isolates, suggesting the relative non-aggressiveness of the response isolate. Based on the obtained *AI* values, we assume that *E. parasitica* is usually a weak competitor compared to other fungal species which are present in the dead branches of *A. pseudoplatanus*. This is consistent with our previous results [27], where the quick progression of *E. parasitica* from the wood of the outer part of the dead branch into the wood of the trunk was observed. However, the outcome of interactions in nature varies depending on the size and quality of the resource, i.e., wood and microclimate [12].

Different interaction types in replicates of the same dual cultures (in our case *Diaporthe* sp. and *N. acerina*) are frequently observed [4,10,38,39]. A very diverse set of bioactive substances may be produced during interspecific mycelial interactions [3,40] and may have an effect on inhibition abilities, pigment production in the interaction zone, colony color, etc. [2,3,13–15,41]. Further studies of secondary metabolites and their role in antagonistic activity toward *E. parasitica* are therefore needed. Studies of interaction patterns in vitro may produce effective strategies for biological control [3], but extrapolating findings from the laboratory to natural habitats should be done carefully. In the natural environment, inhibition would be influenced by a plethora of additional factors, i.e., inoculum potential, germination efficiency, growth rate, substrate utilization patterns, microclimate, etc. [3]. This study can serve as a guide to the possible outcome of different interactions in nature, but these interactions should be further studied in the natural environment, which was beyond the scope of the study.

4.2. Re-Isolations from Dual Cultures

Re-isolations were not always successful from dual culture assays (co-cultures with *F. avenaceum* and *P. incarnata*). If we re-isolated both isolates from the interaction zone, we assumed that the challenge isolates were not very active competitors. In contrast, re-isolation of only the challenge isolate from the interaction zone suggested the elimination of the response isolate of *E. parasitica* and obvious higher success in competition. This was consistent with higher average values of *s*. Negative values of *s* were calculated for co-cultures with *Diaporthe* sp., *N. acerina*, and *Pe. irregularis*, suggesting their weaker competition success.

Re-isolations after deadlock at mycelial contact (interaction type A) resulted mostly in the growth of both isolates, even from the same discs. Koukol, Mrnka, Kulhankova, and Vosatka [32] reported the same re-isolation results after deadlock at a distance (interaction type B), which was not the general case in our study. Re-isolations from assays with interaction type B yielded both isolates in only two out of ten cases, while in the eight other cases, re-isolations yielded only *E. parasitica* colonies. The mycelium of *E. parasitica* remained viable and grew from re-isolation discs even after being partially replaced by *P. incarnata*. In contrast, re-isolations from dual cultures with *Eutypa* sp., *F. avenaceum* and *Neonectria* sp. did not yield any colony of the response isolate from interaction zone, which suggests replacement of the response isolate and its weaker competitive ability against those challenge isolates. The fact that *Eutypa* sp., *F. avenaceum*, and *Neonectria* sp. were able to outcompete the response isolate of *E. parasitica* suggests that *E. parasitica* probably invests less energy in direct mycelial competition against those challenge isolates, as reported by Koukol, Mrnka, Kulhankova, and Vosatka [32]. Although no research has addressed this until now, the re-isolation tests offered a reliable tool to confirm the re-isolation success of an individual isolate after contact with a competitor's mycelium [32].

4.3. Summary of In Vitro Interactions between E. parasitica and Challenge Isolates

Based on *AI*, the inhibition effect of *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* on the response isolate was significantly higher in comparison with other challenge isolates. These results were consistent with the expected very successful re-isolation of these challenge isolates from the interaction zones. Based on *s*, *Eutypa* sp. and *Neonectria* sp. were good inhibitors of *E. parasitica* growth and destroyed it in the interaction zone. Similarly, *Eu. maura* and *P. incarnata* had high *AI* values and achieved relatively high values of *s*. At the interaction zone of those assays, slightly macroscopically changed mycelium was observed, which could be the first sign of response isolate elimination. In contrast, *N. acerina* and *Diaporthe* sp. had relatively low, insignificant values of *AI* and consequently also lower values of *s*, suggesting the higher success of *E. parasitica* in competition with these isolates. Dual cultures of *E. parasitica* and *F. avenaceum* with poor inhibitory effect resulted in poor re-isolation of the response isolate. This is additional proof of the extreme complexity of interspecific interactions, which sometimes result in extraordinary and unexpected outcomes that are of great interest for further research.

The results presented in this study are the first insight into the complex interactions between the maple pathogen *E. parasitica* and some of the most frequently isolated fungal species from the wood of the dead branches of young sycamore maple.

Eutypella canker of maple caused by *E. parasitica* can cause significant economic and resource loss. Biological control of *E. parasitica* would therefore be an excellent way to avoid or at least minimize the negative effects of the pathogen. Preliminary tests of possible antagonism in the laboratory are crucial for achieving a basic understanding of interactions and searching for potential biological control agents. The significance of the obtained results *in vivo* remains to be investigated. The exact microclimate and the whole fungal community present in natural environment were not able to be fully mimicked in the laboratory conditions. Furthermore, studies of secondary metabolites and their role in antagonistic activity would be beneficial.

Finally, we would like to point out a drawback of this study in the light of the interpretation and generalization of the obtained results. Because of the low number of repetitions and only one

tested reference isolate of *E. parasitica*, the results should be treated with caution. Further tests are needed to verify the universality of the obtained findings, with wider range of response isolates of *E. parasitica* tested.

5. Conclusions

The interactions between the ten most frequently isolated species from the wood of the dead branches of *A. pseudoplatanus* and *E. parasitica* in dual cultures on PDA revealed two main types of competitive interactions: Deadlock, consisting of mutual inhibition after mycelial contact or at a distance, and replacement, resulting in the inhibition of *E. parasitica*, followed by partial overgrowth by the replacing fungus. The results of the antagonism index suggested that *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* were the most competitive and had the highest inhibition of *E. parasitica* growth. These isolates are promising candidates for use as biocontrol agents but additional experiments with different *E. parasitica* isolates should be done for confirmation and clarification of our results. Re-isolations revealed that the mycelium of *E. parasitica* at the interaction zones remained mostly viable, except in dual cultures with *Eutypa* sp., *Neonectria* sp., and *F. avenaceum*. Based on the results of the antagonism index (*AI*) and re-isolation success (*s*) in our preliminary study, we can assume that *E. parasitica* is a weak competitor which invests less energy in direct mycelial competition. Nevertheless, additional tests and supplementary experiments with a wider range of *E. parasitica* isolates should be performed for a solid conclusion. The results provide a general insight into the antagonistic activities of the ten most frequently isolated fungi from the wood of the dead branches of *A. pseudoplatanus* against *E. parasitica*, as well as a basis for further research.

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2.3 VPLIV *Eutypella parasitica* NA RAZKROJ LESA TREH VRST JAVORJEV

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Eutypella parasitica R. W. Davidson and R. C. Lorenz je povzročiteljica javorovega raka, uničujoče bolezni javorjev v Evropi in Severni Ameriki. Znano je, da gliva *E. parasitica* povzroča obarvanje in razkroj lesa, vendar ni znano, kako uspešna je pri razkroju najbolj razširjenih vrst javorjev v Evropi. Na podlagi prilagojenega standarda EN 113 smo vzorce lesa *Acer pseudoplatanus* L., *A. platanoides* L. in *A. campestre* za primerjavo izpostavili štirim izolatom *E. parasitica* in devetim drugim glivnim vrstam. Po 15-tedenski inkubaciji sta izguba mase in mikroskopska analiza vzorcev pokazali dobro razraščanje glivnih vrst ter različne potenciale razkroja lesa. Odkrili smo statistično značilno pozitivno povezavo med izgubo mase in vsebnostjo vode pri vseh vrstah gliv. Podobno dobro povezani sta bili tudi izmerjena debelina celičnih sten in izguba mase vzorcev. V povprečju so glive povzročile najmanjšo izgubo mase pri vzorcih *A. pseudoplatanus* (10,0 %) in največjo pri vzorcih *A. campestre* (12,6 %). Med vzorci, ki so bili izpostavljeni izolatom *E. parasitica,* je bila največja izguba mase evidentirana pri *A. pseudoplatanus* (6,6 %). Statistična analiza je pokazala tudi značilne razlike v izgubi mase in vsebnosti vode med različnimi izolati *E. parasitica*. Na podlagi razbarvanja razpravljamo o tipu trohnobe, ki ga povzroča *E. parasitica*. Čeprav so izolati *E. parasitica* povzročili manjšo izgubo mase vzorcev v primerjavi z drugimi, učinkovitejšimi vrstami, ne smemo zanemariti njene sposobnosti razkroja lesa javorjev. Ker *E. parasitica* po navadi okuži nižje dele debla, ki so hkrati najdebelejši in najvrednejši deli drevesa, lahko vsaka tovrstna poškodba povzroči znatno ekonomsko izgubo.

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The Effect of *Eutypella parasitica* on the Wood Decay of Three Maple Species

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Abstract: *Eutypella parasitica* R.W. Davidson & 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* is known to cause wood stain and decay. However, it is not known how effectively it decomposes the wood of the most widespread maple species in Europe. Wood samples of *Acer pseudoplatanus* L., *A. platanoides* L., and *A. campestre* L. were exposed to four isolates of *E. parasitica* and nine other fungal species for comparison, according to the modified EN 113 standard. After 15 weeks of incubation, mass loss and microscopical analysis of samples showed evidence of colonization and different wood decay potentials among fungal species. A highly significant positive correlation was found between mass loss and moisture content for all fungal species. Similarly, the measured cell wall thickness correlated well with the calculated mass loss of the samples. On average, the fungal species caused the lowest mass loss in *A. pseudoplatanus* (10.0%) and the highest in *A. campestre* (12.6%) samples. Among the samples exposed to *E. parasitica* isolates, the highest mass loss was recorded in *A. pseudoplatanus* (6.6%). Statistical analysis showed significant differences in mass loss and moisture content between different *E. parasitica* isolates. Based on the results of staining, we discuss the type of decay caused by *E. parasitica*. Although *E. parasitica* isolates caused smaller mass loss of samples compared to other more effective decay species, we should not disregard its capability of degrading maple wood. Because *E. parasitica* usually infects the lower portion of the trunk, which is the largest and most valuable part of the tree, any damage can cause significant economic and resource loss.

Keywords: wood decay; mass loss; moisture content; mini-block test; decay test; *Acer* spp.; *Eutypella parasitica*; fungi; light microscopy; scanning electron microscopy

1. Introduction

Wood decay is the biological process by which cell wall components (cellulose, hemicellulose, and lignin) are converted to carbon dioxide and water with a release of energy [1,2]. Within a wide spectrum of different types of decay, three main categories are commonly recognized: brown rot, white rot, and soft rot [3]. Different fungi utilize different strategies for degradation and attack the main chemical components of wood [4,5]. When wood-degrading fungi grow through vascular tissues and metabolize wood, a decrease in wood mass and strength usually occurs [2,5]. It is estimated that a 10% loss of wood mass can result in a 70 to 90% loss in wood strength, depending on the wood species

and type of decay [2]. Not all fungal infestations cause degradation [5]. Various host species can be affected differently by the same fungus [6].

Non-basidiomycete fungi (predominantly ascomycete) can cause substantial degradation of wood, but comprehensive studies on the effects of such fungi are rare [7–9]. *Eutypella parasitica* R.W. Davidson & R.C. Lorenz, the causative agent of Eutypella canker of maple, causes a serious disease that affects the aesthetic and economic value of infected maple trees [10]. The fungus is believed to originate from North America [11] and represents a considerable risk for an extensive area of naturally distributed maples in Europe [12]. According to the data of French [13], the decay of *Acer saccharum* Marshall, *A. rubrum* L., and *A. saccharinum* L. wood exposed to *E. parasitica* is extremely slow. His study did not identify differences in the effect of different isolates on the degradation of wood. In general, French [11] recorded greater mass loss in sapwood than in false heartwood.

Based on a literature study, we found that there is no consistency in decay type caused by *Eutypella* species. Worrall, Anagnost and Zabel [7] suggested that *E. parasitica* has the ability to cause soft rot decay. In contrast, data on wood colonization suggest that, in early stages, *Eutypella* species could be considered as a soft rot fungus, but, in more advanced stages, they show a uniform degradation similar to white rot [14]. Pildain, Novas, and Carmarán [14] stated that the type of decay is difficult to define and differentiate because of the difference in observed features in enzyme tests and anatomical studies. After Worrall, Anagnost, and Zabel [7], they suggested the use of the taxonomy of the causal agent as the defining factor in such cases. One of the possible reasons for the observed differences is the fact that the same fungi can cause different types of decay depending on the environmental conditions, predominantly moisture content in the living or dead tree [15].

Eutypella parasitica is known to cause wood stain and decay. However, it is not known how effectively it decomposes the wood of the most widespread European maple species. Therefore, the aim of our study was to determine the mass loss and moisture content of *Acer pseudoplatanus* L., *A. platanoides* L. and *A. campestre* L. samples after exposure to several *E. parasitica* isolates. The results were compared to the impact of five frequently isolated fungal species from the wood of dead branches of *A. pseudoplatanus* [16] and to two well-known basidiomycete decay fungi—*Trametes versicolor* (L.) Lloyd and *Gloeophyllum trabeum* (Pers.) Murrill. In addition, light and scanning electron microscopy were used to examine the differences in wood structure between exposed and control samples of *A. pseudoplatanus*. We expected relatively low mass loss of maple wood due to *E. parasitica* compared to other fungal species included in the experiment since there are at least two (*T. versicolor* and *G. trabeum*) known as significant rot fungi.

2. Materials and Methods

2.1. Decay Test

Three maple species (*Acer pseudoplatanus*, *A. platanoides*, and *A. campestre*) were used in the experiment. The wood originated from the south-eastern part of Slovenia (45.8491° N, 15.6113° E, 390 m a.s.l.), where all three species grow naturally. Cross-sections were taken approximately 1 m above the ground from visually healthy trees. Until further processing they were stored in an LTH ZO 700 BEZ freezer cabinet (Loška hladilna tehnika, Škofja Loka, Slovenia) at −20 °C for 7 to 20 days. One hundred and sixty wood samples (30 × 10 × 5 mm) were made from each maple species. The initial dry mass of the samples was determined using a Kern ABJ220-4NM analytical balance (Kern & Sohn, Balingen, Germany) after oven drying the samples at 103 °C for 24 h in a Kambič SP-250 oven (Kambič, Semič, Slovenia). Thereafter, samples were steam sterilized (30 min, 121 °C, 0.12 MPa) in a Kambič A-65 V autoclave (Kambič, Semič, Slovenia) and further used in the decay test.

The decay test was performed according to the modified EN 113 standard [17–20]. Disposable Petri dishes (Ø = 90 mm, h = 15 mm) containing 3.9% (*w/v*) potato dextrose agar (PDA; Becton Dickinson, Sparks, MD, USA) were inoculated with ten different fungal species (Table 1). After one week of fungal growth, the wood samples were exposed to the different fungi. Two wood samples were placed on a

sterilized plastic mesh, which was used to avoid direct contact between the samples and the nutrient medium in the Petri dish. Ten replicates per fungal isolate were used for each of the three maple species (450 samples in total). For comparison, ten control samples per maple species were placed in Petri dishes with sterile agar plugs. The assembled test dishes were incubated in a Kambič I-190 CK chamber (Kambič, Semič, Slovenia) at 23.9 ± 0.3 °C for 15 weeks. After incubation, the fungal mycelium was carefully removed from the samples, and they were weighed to obtain the mass of the wet sample. After 24 h of drying at 103 °C in a Kambič SP-250 oven (Kambič, Semič, Slovenia), the final dry mass was determined and the respective relative loss in mass was calculated using Equation (1). From the final dry and wet mass, the moisture content was calculated using Equation (2).

$$\textit{Mass loss}\ (\%) = ((m_0 - m_2)/m_0) \times 100, \quad (1)$$

$$\textit{Moisture content}\ (\%) = ((m_1 - m_2)/m_2) \times 100, \quad (2)$$

where m_0 represents the initial dry mass, m_1 the final wet mass, and m_2 the final dry mass of the sample. Furthermore, the change in moisture content (ΔMC) was calculated using the following equation:

$$\Delta MC\ (\%) = MC_E - MC_C, \quad (3)$$

where MC_E represents the average moisture content of samples exposed to the fungal species, and MC_C is the average moisture content of the control samples.

Table 1. Wood decay fungi used in decay tests.

Fungi	Label [5]	Collection Number [6]
Diaporthe sp. Nitschke [1]	Ds	ZLVG 788
Eutypa sp. Tul. & C. Tul. [1]	Es	ZLVG 790
Eutypa maura (Fr.) Sacc. [1]	EM	ZLVG 789
Eutypella parasitica R.W. Davidson & R.C. Lorenz	EP34	ZLVG 34
	EP65	ZLVG 65
	EP67	ZLVG 67
	EPT	ZLVG 805
Fusarium sp. Link [1]	Fs	ZLVG 792
Gloeophyllum trabeum (Pers.) Murrill [2]	GT	ZIM L017
	GTD	DSM 1398
Neocucurbitaria acerina Wanas., Camporesi, E.B.G. Jones & K.D. Hyde [1]	NA	ZLVG 794
Neonectria faginata (M.L. Lohman, A.M.J. Watson & Ayers) Castl. & Rossman [3]	NF	ZLVG 807
Neonectria punicea (J.C. Schmidt) Castl. & Rossman [3]	NP	ZLVG 806
Trametes versicolor (L.) Lloyd [4]	TV	ZIM L057
	TVD	DSM 3086

[1] The most frequently isolated species from the wood of the dead branches of *A. pseudoplatanus* [16]; [2] brown rot; [3] co-isolated from Eutypella canker of maple; [4] white rot; [5] labels used throughout the text; [6] ZLVG—Culture collection of the Laboratory of Forest Protection at the Slovenian Forestry Institute; ZIM—Culture collection of industrial microorganisms at the University of Ljubljana; DSM—German collection of microorganisms and cell cultures at the Leibnitz Institute.

A non-parametric Kruskal-Wallis test was used to compare average mass loss and moisture content between different isolates. Afterwards, a post-hoc multiple comparison Dunn test with Bonferroni correction was used. A paired *t*-test was used to compare the initial and final mean dry mass of samples exposed to different fungal isolates. For comparing mass loss and moisture content between the three maple species, the Welch two sample *t*-test was used. The Pearson correlation coefficient was calculated to investigate the relationship between mass loss and moisture content in all three maple species.

Additionally, the wood density of the samples was calculated using the equation:

$$\textit{Wood density}\ (\mathrm{kg/m^3}) = m_0/V, \quad (4)$$

where m_0 represents the initial dry mass of a sample, and V is the volume of a single sample (1.5×10^{-6} m^3 in our case). A non-parametric Kruskal-Wallis test with a post-hoc multiple comparison Dunn test with Bonferroni correction was used to compare the average wood density between the different maple species. The Pearson correlation coefficient was calculated to investigate the relationship between moisture content and wood density in all three maple species. The calculated average mass loss and average wood density were discussed and compared to other relevant studies.

2.2. Light and Scanning Electron Microscopy

Three randomly chosen wood samples (one per fungal isolate) of *A. pseudoplatanus* exposed to EP34, EP65, and TV were examined and compared to the control using light microscopy (LM) and scanning electron microscopy (SEM). For LM, halved samples were embedded in paraffin (Paraplast Plus, Leica Biosystems, Wetzlar, Germany) and cut with a Leica RM2245 rotary microtome (10 µm; Leica Biosystems, Wetzlar, Germany). Cross and tangential sections were stained with a safranin (Safranin T, Honeywell Fluka, Thermo Fisher Scientific, Waltham, Massachusetts, United States) (0.04%) and astra-blue (Astrablau FM, Carl Roth, Karlsruhe, Germany) (0.15%) water solution [21]. Safranin colors the polyphenol components, such as lignin, red, whereas astra-blue stains the cellulose/hemicellulose components of cell walls blue [22,23]. The sections were mounted on glass slides in Euparal (Waldeck, Münster, Germany), observed and photographed under a Leica DM4000 B light microscope with a Leica DCM 4500 camera and the Leica Application Suite software platform (Leica Microsystems, Wetzlar, Germany).

Wood colonization was also examined with SEM, using an Everhart-Thornley (ETD) detector in an FEI Quanta 250 scanning electron microscope (FEI, Hillsboro, Oregon, United States) at a working distance of between 7 and 9 mm and 1000× magnification. Prior to observation, samples were halved and smoothed at cross and tangential surfaces with a Leica SM2010R sliding microtome (Leica Biosystems, Wetzlar, Germany), adhered to the holder with carbon adhesive tabs and coated with Au/Pd sputter-coater (Q150R ES Coating System, Quorum technologies, Laughton, UK) for 30 s with 20 mA intensity. SEM micrographs were visualized with xT microscope control software v 6.2.11 build 3381 (Microsoft Corporation, Redmond, Washington, United States).

Histometric analysis of wood features was performed on cross-sections under an Olympus BX51 microscope (Olympus, Tokyo, Japan) with a Nikon Digital Sight DS-Fi1 camera (Nikon, Kanagawa, Japan) and NIS Elements BR software version 3.22.09 build 726 (Nikon, Tokyo, Japan). At 40× objective magnification, we measured the cell wall thickness (µm) of 30 randomly chosen fibers in the early, transition and late wood of *A. pseudoplatanus*. Additionally, 10 randomly chosen vessels were measured in the early, transition, and late wood of the same cross-sections. In total, the cell wall thickness of 90 fibers and 30 vessels was measured on cross-sections from each randomly chosen wood sample exposed to three fungal isolates and a control.

The Kruskal–Wallis test and a post-hoc Dunn test with Bonferroni correction were used to compare average fiber wall thickness between the three different parts of the wood increment (i.e., early, transition, and late wood) and between different isolates. For vessel wall thickness, an ANOVA and Tukey multiple comparison test was used. ANOVA with a post-hoc Tukey HSD test was used when the assumptions of normal distribution and homogeneity of variances had been met. In other cases, the non-parametric Kruskal-Wallis with a post-hoc Dunn test was used. To test the assumption of normality, the Shapiro-Wilk test was applied, and for testing the homogeneity of variances, the Levene test was used. In addition, the Pearson correlation coefficient was calculated to investigate the relationship between mass loss on the one hand, and average fiber and average vessel wall thickness on the other.

All calculations and statistical tests were performed in Microsoft Excel version 1908 and the R software environment for statistical computing [24] with the "car" [25], "DescTools" [26], "multcomp" [27], "dplyr" [28], and "broom" packages [29].

3. Results

3.1. Mass Loss

The mass loss of wood after 15 weeks averaged 10.0% in *A. pseudoplatanus*, 10.7% in *A. platanoides*, and 12.6% in *A. campestre* samples. The results of the non-parametric statistical test (Kruskal-Wallis) revealed significant differences ($p < 0.001$) in the average mass loss of samples between different isolates in all three maple species. Pairwise comparisons of mass loss in *A. pseudoplatanus* revealed statistically significant differences ($p < 0.01$) between the control and Ds, EP65, EP67, EPT, GTD, TV, and TVD. Samples of *A. platanoides* showed similar results, with the exception of EP67, which was not significantly different from the control, and Es, which was significant ($p < 0.01$). Mass loss of control *A. campestre* samples was significantly different ($p < 0.05$) from Es, EP65, EP67, EPT, GT, GTD, TV, and TVD, but not from Ds, EM, EP34, Fs, NA, NF, and NP ($p > 0.05$).

Both isolates of *T. versicolor* (TV and TVD) were the most effective, causing mass loss ranging from 33.1% in *A. pseudoplatanus* to 60.8% in *A. campestre* after 15 weeks of exposure. Similarly, isolates of *G. trabeum* (GT and GTD) caused average mass loss of 12.1% in *A. pseudoplatanus* (Figure 1), 20.6% in *A. platanoides* (Figure 2), and 24.3% in *A. campestre* (Figure 3). The other isolates were less effective, causing mass loss ranging from 1.3 to 11.2% in *A. pseudoplatanus* (Figure 1), 0.4 to 13.1% in *A. platanoides* (Figure 2), and 2.1 to 8.3% in *A. campestre* (Figure 3). The lowest mass loss in *A. pseudoplatanus* samples was obtained for EM, in *A. platanoides* for Fs and in *A. campestre* for NP. The most effective fungi among the five most frequently isolated species from the wood of the dead branches of *A. pseudoplatanus* were Ds in the case of *A. pseudoplatanus* and *A. platanoides* samples, and Es in the case of *A. campestre*.

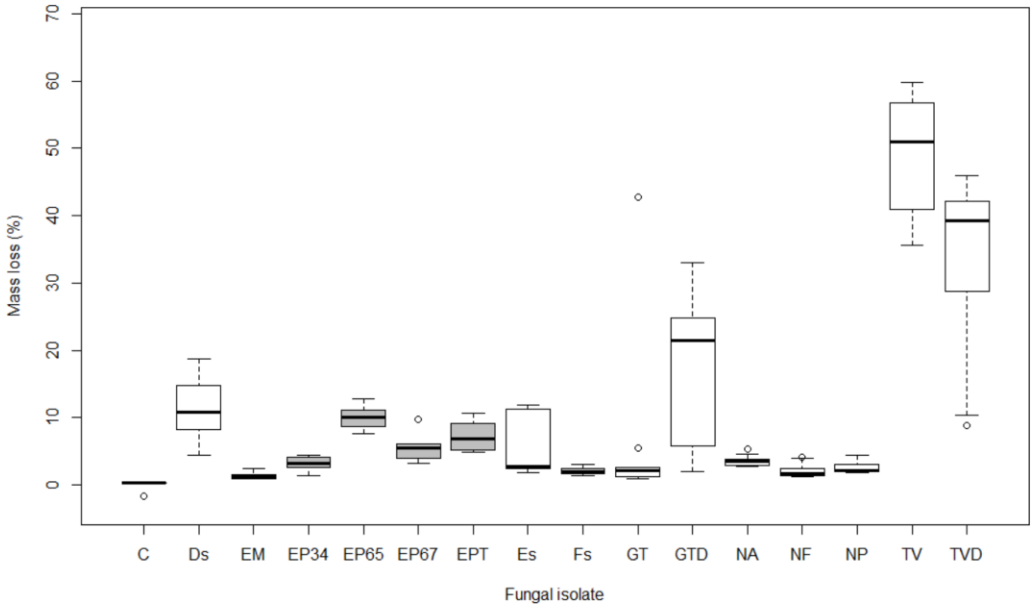

Figure 1. Boxplot showing mass loss (%) after 15 weeks of exposure to different fungal isolates in *Acer pseudoplatanus* (see Table 1 for labels of isolates; boxes of *E. parasitica* isolates are grey). Boxplot represents minimum, first quartile (Q_1), median, third quartile (Q_3), and maximum value.

Differences between average initial and final dry mass were significant for all isolates ($p < 0.05$), except for the controls in all three maple species and GT isolate in *A. pseudoplatanus*.

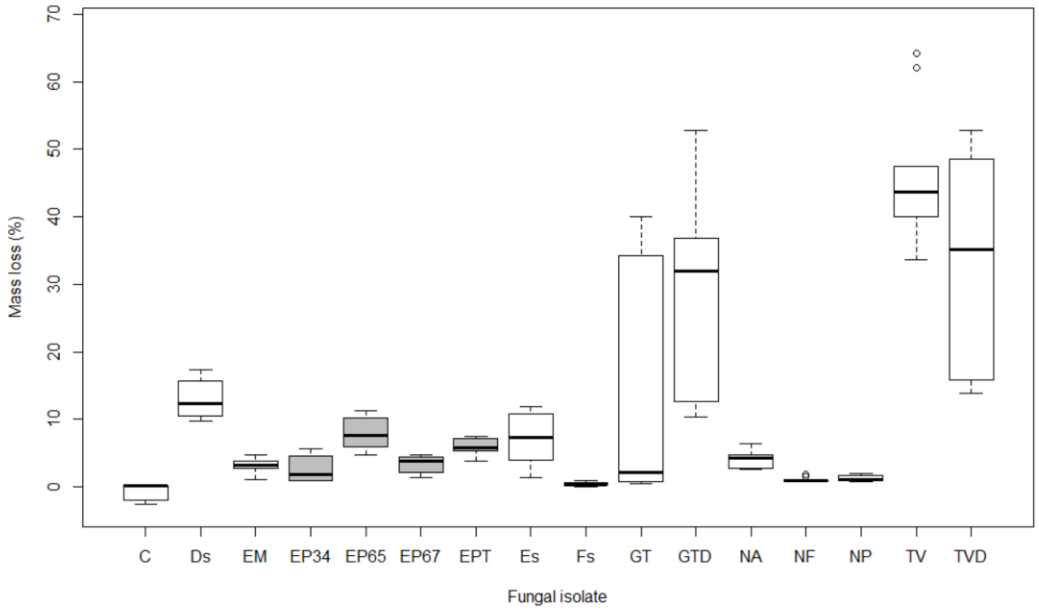

Figure 2. Boxplot showing mass loss (%) after 15 weeks of exposure to different fungal isolates in *Acer platanoides* (see Table 1 for labels of isolates; boxes of *E. parasitica* isolates are grey). Boxplot represents minimum, first quartile (Q_1), median, third quartile (Q_3), and maximum value.

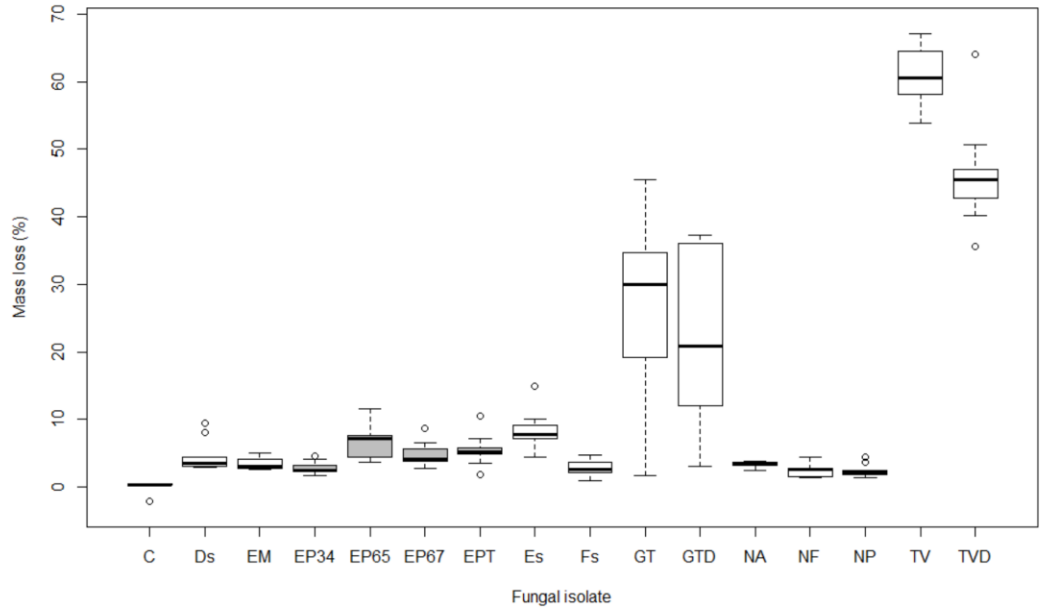

Figure 3. Boxplot showing mass loss (%) after 15 weeks of exposure to different fungal isolates in *Acer campestre* (see Table 1 for labels of isolates; boxes of *E. parasitica* isolates are grey). Boxplot represents minimum, first quartile (Q_1), median, third quartile (Q_3), and maximum value.

Pairwise comparisons between different isolates of *E. parasitica* revealed significant differences in average mass loss between EP34 and EP65 ($p < 0.001$), and between EP34 and EPT ($p < 0.05$), in all three tested maple species. Additionally, there were statistically significant differences between EP65 and EP67 in *A. pseudoplatanus* and *A. platanoides* ($p < 0.05$), and between EP67 and EPT in *A. platanoides* ($p < 0.05$). EP65 was the most effective in causing mass loss, followed by EPT, EP67, and EP34. This was

the case in all three *Acer* species. The highest mass loss was determined for *A. pseudoplatanus* wood samples exposed to *E. parasitica* isolates (average 6.6%). In *A. platanoides* and *A. campestre*, *E. parasitica* isolates were less effective, causing an average mass loss of 4.9%. There were significant differences in average mass loss caused by *E. parasitica* isolates EP65 and EP67 between *A. pseudoplatanus* and *A. platanoides* ($p < 0.05$), and in the average mass loss of *A. pseudoplatanus* and *A. campestre* samples exposed to EP65 ($p < 0.01$).

3.2. Moisture Content

The average moisture content of samples exposed to fungi was 111.8% in *A. pseudoplatanus*, 107.7% in *A. platanoides* and 113.3% in *A. campestre*. Control samples had significantly lower values (57.2% on average) ($p < 0.05$). There were significant differences ($p < 0.01$) in the average moisture content of the control samples in comparison to samples exposed to GT, GTD, TV, and TVD in all tested maple species. Differences from control were also significant for EM, Fs, and NA in *A. pseudoplatanus*, and for EP65 in *A. campestre* ($p < 0.05$). In contrast, the average moisture content of samples exposed to Ds, Es, EP34, EP67, EPT, NF, and NP was not significantly different from control samples in all three maples ($p > 0.05$). In addition to these fungal isolates, the moisture content of samples exposed to EM, Fs, and NA in *A. platanoides* and *A. campestre*, and EP65 in *A. pseudoplatanus* and *A. platanoides*, were also not significant.

The average moisture content of samples exposed to *E. parasitica* isolates was the lowest in *A. pseudoplatanus* at 83.1% (Figure 4), followed by *A. platanoides* at 86.5% (Figure 5) and *A. campestre* at 97.1% (Figure 6). No statistical differences were found between the average moisture content of *A. pseudoplatanus* samples exposed to different *E. parasitica* isolates. Samples of *A. platanoides* exposed to EPT showed statistically significant differences in average moisture content in comparison with EP34 ($p < 0.01$), EP65, and EP67 ($p < 0.05$). In *A. campestre*, we found only one significant pairwise comparison, i.e., between EP34 and EP65 ($p < 0.05$). No statistically significant differences were found between the average moisture content of *A. pseudoplatanus* and *A. platanoides* samples exposed to different *E. parasitica* isolates ($p > 0.05$), but there was a statistically significant difference ($p < 0.05$) in average moisture content between *A. pseudoplatanus* and *A. campestre* exposed to EPT, and between *A. platanoides* and *A. campestre* exposed to EP65 and EPT.

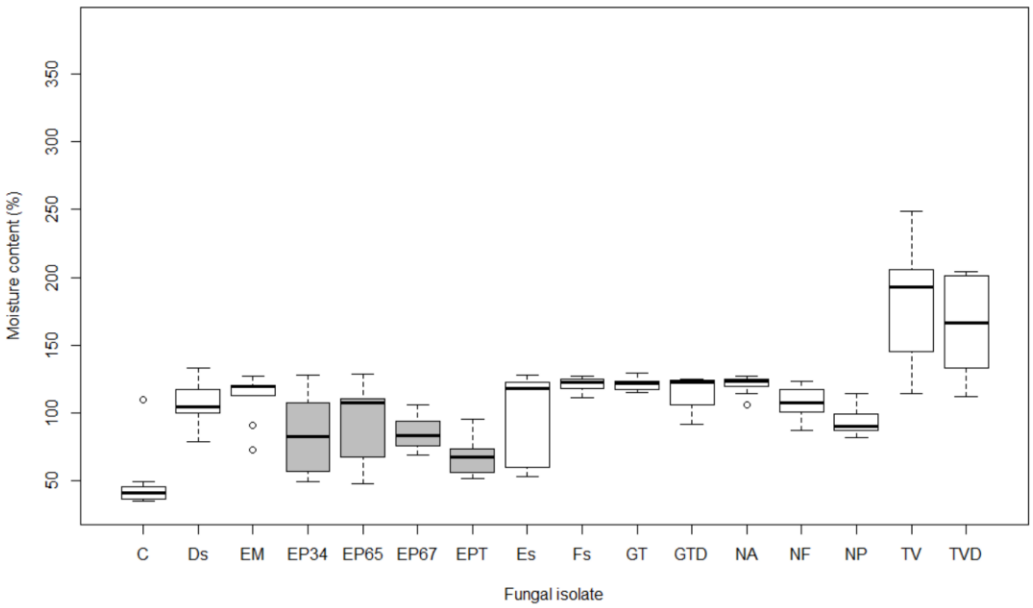

Figure 4. Boxplots showing moisture content (%) after 15 weeks of exposure to different fungal isolates in *Acer pseudoplatanus* (see Table 1 for labels of isolates; boxes of *E. parasitica* isolates are grey). Boxplot represents minimum, first quartile (Q_1), median, third quartile (Q_3), and maximum value.

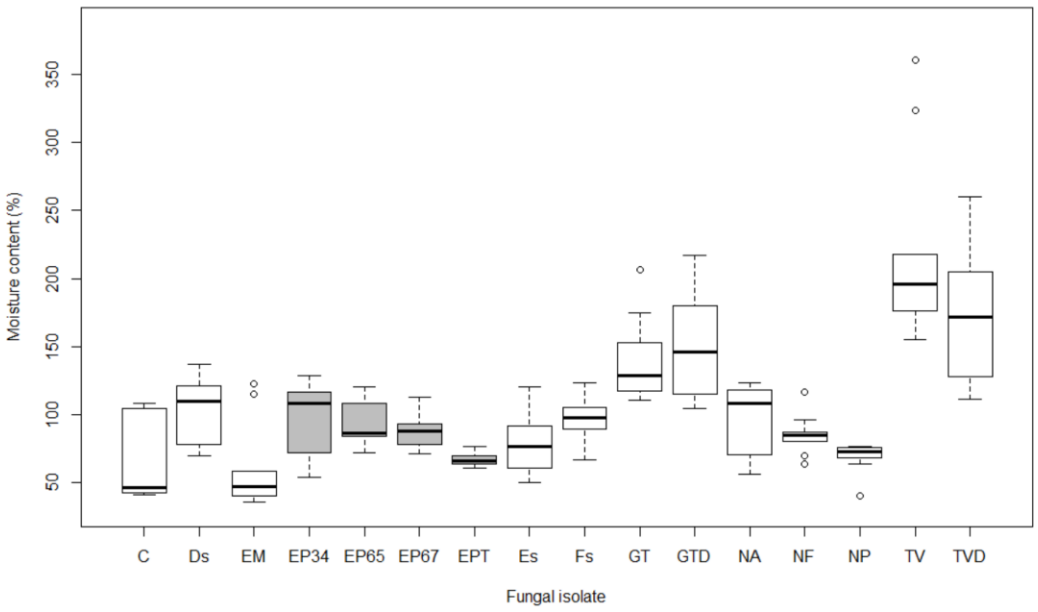

Figure 5. Boxplots showing moisture content (%) after 15 weeks of exposure to different fungal isolates in *Acer platanoides* (see Table 1 for labels of isolates; boxes of *E. parasitica* isolates are grey). Boxplot represents minimum, first quartile (Q_1), median, third quartile (Q_3), and maximum value.

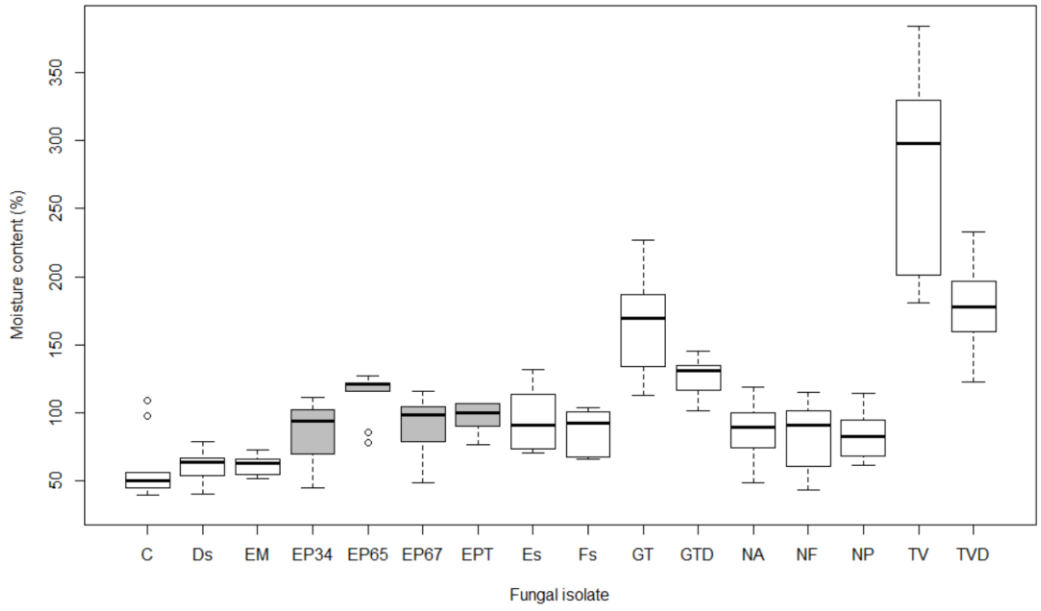

Figure 6. Boxplots showing moisture content (%) after 15 weeks of exposure to different fungal isolates in *Acer campestre* (see Table 1 for labels of isolates; boxes of *E. parasitica* isolates are grey). Boxplot represents minimum, first quartile (Q_1), median, third quartile (Q_3), and maximum value.

The change in average moisture content (ΔMC) between samples exposed to fungal activity and control samples was positive for all fungal isolates and all maple species, except for *A. platanoides* exposed to EM, which showed a negative value (Table 2).

Table 2. Change in average moisture content (Δ*MC*) of samples.

	***A. pseudoplatanus* ΔMC (%)**	***A. platanoides* ΔMC (%)**	***A. campestre* ΔMC (%)**
Diaporthe sp.	58.7	39.3	2.3
Eutypa sp.	53.8	13.9	35.4
Eutypa maura	65.2	−4.3	2.8
Eutypella parasitica [1]	35.7	21.5	38.1
Fusarium sp.	73.6	30.2	27.9
Gloeophyllum trabeum [1]	71.0	78.8	86.5
Neocucurbitaria acerina	73.7	33.3	29.0
Neonectria faginata	60.0	20.0	23.7
Neonectria punicea	45.7	4.1	24.7
Trametes versicolor [1]	125.0	129.8	171.6
Average	64.4	42.6	54.3

[1] Average values for all isolates of certain fungal species.

We found a highly significant ($p < 0.001$) positive correlation between mass loss and moisture content in all three maple species (*A. pseudoplatanus* r = 0.66, *A. platanoides* r = 0.84, and *A. campestre* r = 0.87).

3.3. Wood Density

An average wood density of 616 kg/m^3 was calculated for the *Acer* spp. samples in our study. A significant difference was found with the Kruskal-Wallis test ($p = 0.012$) for the average wood density of the three maple species in the experiment. Only the wood density of *A. campestre* was significantly higher than that of *A. platanoides*. Other pairwise comparisons were not significant (Table 3). References on *Acer* spp. wood density usually report an average of 640 kg/m^3 for *A. pseudoplatanus* and 590 kg/m^3 for *A. platanoides* and *A. campestre* [30]. Furthermore, we did not find a statistically significant correlation ($p > 0.05$) between the moisture content and wood density of samples in any of the maple species.

Table 3. Average wood density and standard deviation of three maple species.

Tree Species	Wood Density (kg/m^3)	SD [1]	Statistic Group [2]
Acer pseudoplatanus	616	18	ab
Acer platanoides	611	26	b
Acer campestre	620	29	a

[1] SD—standard deviation. [2] Different letters indicate significant differences.

3.4. Light and Scanning Electron Microscopy

Light microscopy (LM) of the wood samples of *A. pseudoplatanus* exposed to EP34, EP65, and TV confirmed the obvious colonization and also occasional degradation of woody tissues. Vessels in wood samples exposed to EP65 and TV were completely colonized (Figure 7e,f,g,h), while, in samples exposed to EP34, the presence of fungal hyphae was not so abundant and, in most cases, limited to the edge of the sample (Figure 7c,d). Samples exposed to EP65 showed some parts with fiber wall thinning and abundant hyphae in the vessels (Figure 7e,f). In contrast, almost no changes in cell wall color, cell wall thickness and evidence of fungal hyphae were observed in samples exposed to EP34 (Figure 7c,d). The LM images confirmed that the wood samples exposed to TV suffered the greatest cell wall degradation in *A. pseudoplatanus*. Cell wall thinning, sometimes larger parts of destroyed cell walls, and vessel lumens filled with fungal hyphae were observed in those wood samples (Figure 7g,h).

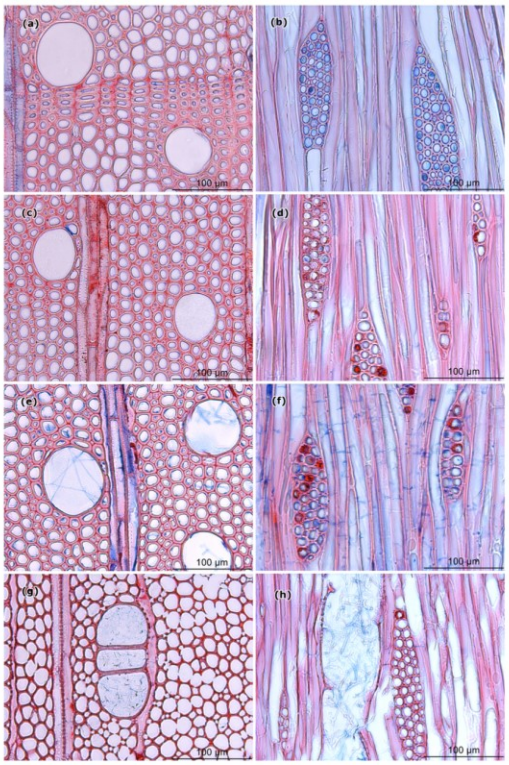

Figure 7. Light microscopy (LM) images of cross and tangential sections of (**a**,**b**) control samples and wood samples after 15 weeks of exposure to (**c**,**d**) EP34, (**e**,**f**) EP65, and (**g**,**h**) TV.

The scanning electron micrographs (Figure 8) confirmed that the 15 weeks of exposure of the wood to fungi caused significant changes to the cell walls. The most severe damage was observed in samples exposed to TV (Figure 8g,h), which also had the highest mass loss. In general, scanning electron microscopy (SEM) revealed thinning and a torn appearance of the fiber wall in degraded cells. The damage was lower in EP65 (Figure 8e,f), and almost no damage was observed in EP34 (Figure 8c,d). Micrographs of EP34 revealed the presence of fungal hyphae, which were hardly observed under LM (Figure 7c,d).

In total, the thickness of 360 fiber walls and 120 vessel walls was measured. Average fiber and vessel wall thickness (Figures 9 and 10) decreased with increasing fungal activity and mass loss. A non-parametric Kruskal-Wallis test and ANOVA revealed statistically significant differences ($p < 0.001$) in average fiber and vessel wall thickness between samples exposed to different fungal isolates. Significant differences ($p < 0.001$) were found for average fiber wall thickness in pairs: TV-control, TV-EP34 and TV-EP65. Similarly, average vessel wall thickness differed significantly between samples exposed to TV and the control ($p < 0.001$), TV and EP34 ($p < 0.05$), and TV and EP65 ($p = 0.05$). Pairwise comparison showed significant differences in average fiber wall thickness between TV and all other fungal isolates in all three parts of the wood increment (i.e., late wood, transition wood, and early wood). Average vessel wall thickness among samples exposed to different fungal isolates was significantly different only in transition wood and early wood. In both parts of the wood increment, differences were significant between samples exposed to TV and the control ($p < 0.05$ in transition and $p < 0.01$ in early wood). In transition wood, TV and EP34 also differed slightly significantly ($p = 0.06$). No statistical differences ($p > 0.1$) were found in average fiber and vessel wall thickness between the control and EP34 and EP65 in all three parts of the wood increment.

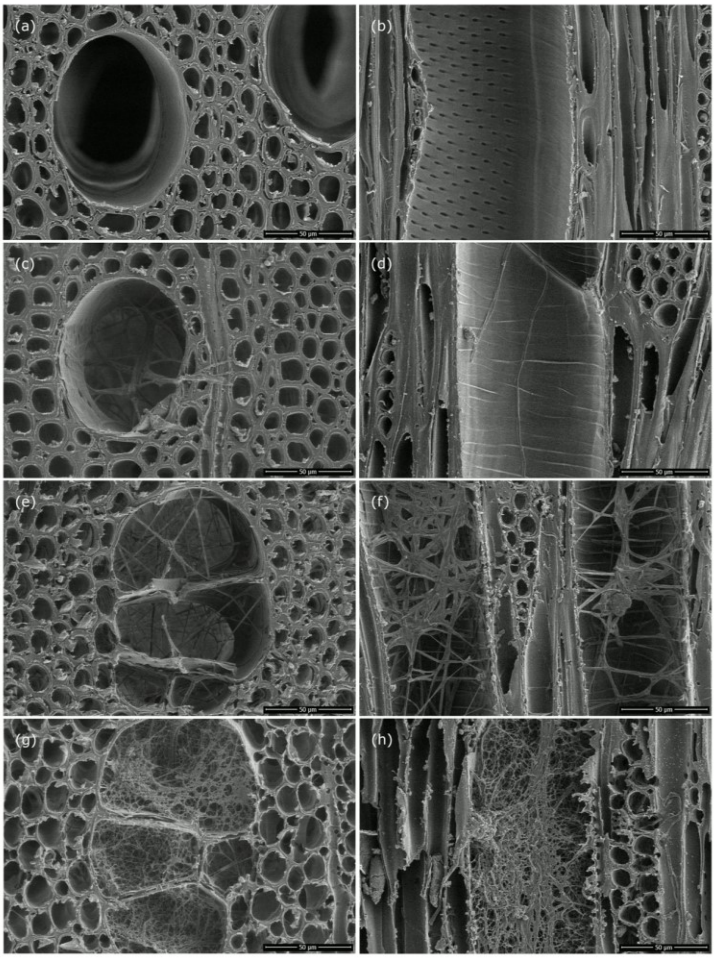

Figure 8. Scanning electron microscopy images of cross and tangential sections of (**a**,**b**) control samples and wood samples after 15 weeks of exposure to (**c**,**d**) EP34, (**e**,**f**) EP65, and (**g**,**h**) TV.

Tests for differences in cell wall thickness between different parts of wood in the samples exposed to the same fungal isolate showed no statistical significance in the vessels. This was not the case for fiber wall thickness, which revealed significant differences between late wood and transition wood for TV ($p < 0.001$) and EP65 ($p < 0.01$), between late wood and early wood for TV, EP34, EP65 ($p < 0.001$), and the control ($p < 0.05$), and finally between transition wood and early wood for EP34 ($p < 0.001$) and TV ($p < 0.05$).

We found a highly significant ($p < 0.001$) negative correlation between mass loss, on the one hand, and average fiber and average vessel wall thickness on the other. The Pearson correlation coefficient between mass loss and average fiber wall thickness was −0.83, and between mass loss and average vessel wall thickness −0.64.

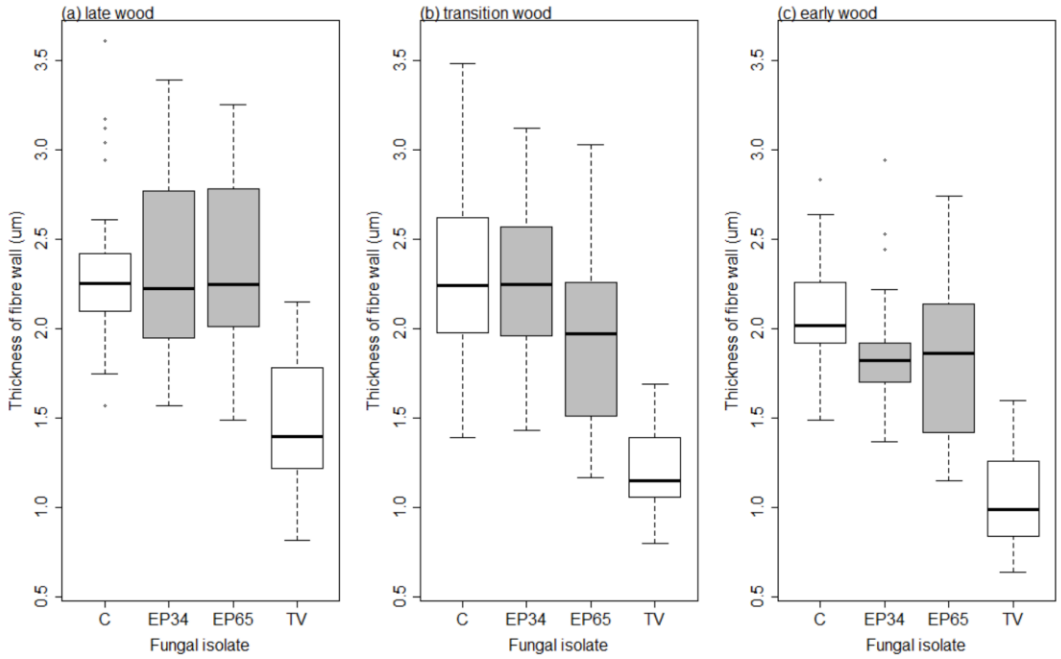

Figure 9. Fiber wall thickness (µm) in (**a**) late wood, (**b**) transition wood, and (**c**) early wood after exposure to different fungal isolates (see Table 1 for labels of isolates; boxes of *E. parasitica* isolates are grey). Boxplot represents minimum, first quartile (Q_1), median, third quartile (Q_3), and maximum value.

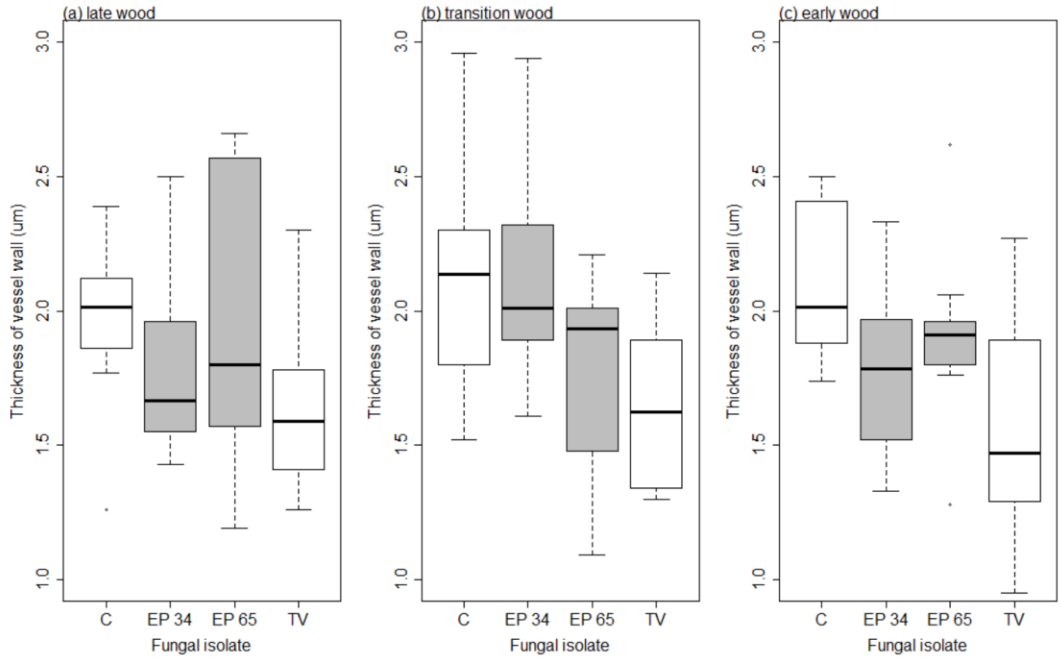

Figure 10. Vessel wall thickness (µm) in (**a**) late wood, (**b**) transition wood, and (**c**) early wood after exposure to different fungal isolates (see Table 1 for labels of isolates; boxes of *E. parasitica* isolates are grey). Boxplot represents minimum, first quartile (Q_1), median, third quartile (Q_3), and maximum value.

4. Discussion

4.1. Mass Loss

All fungal isolates caused greater mass loss compared to control samples, but not all comparisons were significant. Fifteen weeks of exposure to ten different fungal species resulted in notable mass loss of *A. pseudoplatanus*, *A. platanoides*, and *A. campestre* wood samples. The mass loss of samples exposed to EN 113 [20] standard fungi *T. versicolor* was higher than 25%. This clearly indicates that the fungal mycelia were vital and that the test was performed correctly. The mass loss of samples exposed to EM (in *A. pseudoplatanus*), EP34 (in *A. platanoides* and *A. campestre*), Fs, NF, and NP was lower than 3% and thus considered insignificant according to EN 113 [20].

As expected, *T. versicolor* and *G. trabeum* were the most efficient species in the colonization and decay of wood samples. Both species are known as quick colonizers and efficient decay organisms of different woody species worldwide [31,32]. Average mass loss caused by *T. versicolor* isolates in samples of *A. pseudoplatanus* and *A. platanoides* (40.9% and 40.0%) in our study was almost two times higher than previously reported by Reinprecht, Vidholdová, and Gašpar [33] (20.4% in *A. pseudoplatanus*) and Kielmann, Adamopoulos, Militz, and Mai [34] (32.0% in *A. platanoides*). Differences in mass loss between studies could be ascribed to shorter or longer exposure times (6 weeks in Reinprecht, Vidholdová, and Gašpar [33] and 16 weeks in Kielmann, Adamopoulos, Militz, and Mai [34]), fungal isolate origin, vitality and strength, dimensions of samples, eventual fungal combinations, incubation temperature and humidity, agar media, etc. However, our results clearly indicate the low durability of maple wood and high degrading ability of the respective white rot fungi.

The average mass loss of samples exposed to GT isolate indicate the possibility of the unsuccessful inoculation of this isolate in *A. pseudoplatanus* and partly also in *A. platanoides* samples. The difference between average mass loss caused by GT and GTD was simply too large, although these are just two isolates of the same fungal species. Our suspicion was also confirmed with the non-significant difference between the average initial and final dry mass of *A. pseudoplatanus* samples exposed to GT and the non-significant difference in the average mass loss of control samples and samples exposed to GT in *A. pseudoplatanus* and *A. platanoides*. However, the moisture content after 15 weeks of exposure to GT in *A. pseudoplatanus* samples clearly showed some fungal activity.

Our results on mass loss caused by *E. parasitica* isolates are somewhat similar to those reported by French [13], who obtained an average mass loss of 1.13, 1.35, and 1.38% in the false heartwood of red, sugar and silver maple, respectively. French [13] reported a slightly higher average mass loss in the sapwood of the same tested tree species—1.28% in red maple, 3.68% in sugar maple, and 2.44% in silver maple. In contrast with our results, French [13] reported no significant differences in average mass loss between five isolates of *E. parasitica*. Besides the study of French [13], Pildain, Novas, and Carmarán [14] and Worrall, Anagnost, and Zabel [7] also used *Eutypella* spp. or *E. parasitica* isolates in their decay tests. The former studied the wood decay of *Populus deltoides* W. Bartram ex Marshall in a 12-week experiment with four isolates of *Eutypella* spp. and calculated an average mass loss of 17.6%. The latter exposed *Betula alleghaniensis* Britt. and *Pinus taeda* L. sapwood to *E. parasitica* and reported 5.6% mass loss for *Betula* and 2.3% for *Pinus*. When comparing mass loss caused by *E. parasitica* isolates, it should be noted that the methodologies used in foreign studies [7,13,14] were slightly different from the methodology used in our study. Therefore, direct comparisons of the results are not appropriate. However, the present study provided an overview of the degradation activity and strength of *E. parasitica* on *Acer* spp. As expected, the rate and degree of wood degradation by *E. parasitica* isolates was slower and lower in comparison with *T. versicolor* and *G. trabeum*.

4.2. Moisture Content

The overall moisture content of the samples in our study was rather high. As the samples were not in direct contact with the nutrient medium, it can be presumed that the high moisture content was the result of fungal activity. Although the moisture content was rather high for classical wood

degrading fungi [35], it should be noted that the fungi used in our study predominantly colonize living trees and are thus adapted to higher moisture content [36]. Fungi that cause mass loss produce water. In theory, the complete decomposition of 1 g of cellulose by microbial action liberates 0.555 g of water [37]. Thus, it is difficult to say whether lower mass loss is a result of lower wood moisture content or lower moisture content is a result of lower mass loss [38]. Zelinka, Kirker, Bishell, and Glass [38] have suggested examination of the extra effect of fungal growth on the final moisture content by computing the change in moisture content. ΔMC should represent the extra amount of moisture generated by the fungus. Consistent with Zelinka, Kirker, Bishell, and Glass [38], our results showed that higher amounts of fungal metabolism, which are reflected in higher mass loss, are associated with higher final moisture content (especially true for *T. versicolor* and *G. trabeum* isolates). The reason for the negative value of ΔMC for EM in samples of *A. platanoides* is not clear, but other authors have also reported this phenomenon [38,39].

The non-statistically significant differences in average moisture content between control samples and samples exposed to certain fungi suggest that those fungal species did not produce high amounts of water, which is consistent with their lower decomposition ability.

The calculated mass loss correlated well with the calculated moisture content of the wood samples. A positive linear trend can be observed for the relationship between mass loss and moisture content, as already described in the literature [35,38].

4.3. Wood Structural Changes

Microscopy revealed additional information on wood sample colonization and confirmed that mass loss was the consequence of fungal activity. The tested fungi colonized the vessels, which are the widest cells in maple wood and represent a kind of highway for fungi, as reported by Humar, et al. [40] for oak wood. In our study, TV was the most efficient fungus in *A. pseudoplatanus* colonization and decay. The light microscopy images and measurements of cell wall thickness showed that samples exposed to TV suffered significant cellulose and hemicellulose degradation, thus confirming the high susceptibility of sycamore maple wood to this white rot fungus. Severe damage caused by TV attack was further evidenced and confirmed with scanning electron microscopy.

Other fungi included in the SEM analysis were less effective—they were able to colonize maple wood but not able to decompose it to a large extent. Cross and tangential sections of wood samples exposed to EP34 and EP65 showed a considerably smaller impact of the tested fungi on wood degradation. This is consistent with the results of lower mass loss and thicker cell walls compared to well degraded wood samples exposed to TV. However, average fiber and vessel wall thickness in samples exposed to EP34 and EP65 were not significantly different in any part of the wood increment. In contrast to French [13], who reported no difference in average mass loss between different isolates of *E. parasitica*, we detected a significant difference in average mass loss between EP34 and EP65. The differences between *E. parasitica* isolates in our study could be the first sign of possible introduction of the pathogen to North America from Europe, and not the inverse, as is commonly believed. However, such conclusions are speculative and would require a complete study of phylogeny to confirm.

Based on the results of sections stained with safranin/astra-blue water solution, which colored the inner parts of the secondary cell wall slightly blue, we assumed that *E. parasitica* was able to degrade lignin and therefore could be considered as a white rot fungus. Our conclusion about the type of decay caused by *E. parasitica* is consistent with Pildain, Novas, and Carmarán [14], who stated that this fungus could be considered as a white rot fungus in the more advanced stages of wood colonization and as a soft rot fungus in the early stages, as already suggested by Worrall, Anagnost, and Zabel [7]. In general, ascomycete fungi are mainly characterized as soft rot fungi [9], which are able to degrade both polysaccharides and lignin, but lignin is degraded at a later stage [41]. Thus, our suspicion about the type of decay is consistent with past studies. However, such conclusions are somewhat speculative since *E. parasitica* is not very aggressive in wood decay. For the exact determination of the type of decay,

additional analysis of enzyme activity in different environmental conditions should be performed, which was beyond the scope of the present study.

Abundant hyphae in the center of exposed samples, where cross and tangential sections for microscopy were taken, confirmed good overgrowth and successful penetration of fungal mycelium into woody tissue. Higher hyphae abundance suggests higher colonization success and subsequent wood decay by fungi. In the case of wood samples exposed to EP34, where fungal hyphae were more or less limited to the edge of samples, we assumed that the fungus had not effectively progressed into the central part because of the lower isolate ability to penetrate the wood and short time of exposure. French [13] reported penetration of *E. parasitica* into the fibers and vessels, and only occasionally into the ray parenchyma. French [13] did not notice any evident staining or discoloration associated with the decay, which was also the case in our study. In samples exposed to EP34, there was minor sign of fungal activity observed with light microscopy. However, scanning electron microscopy revealed the presence of fungal hyphae in the vessels, indicating and confirming the higher sensitivity of the latter technique. Although light microscopy permits the rapid view of many cells with easier sample preparation, it is better to use it in combination with scanning electron microscopy for reliable detection of fungal presence [42], especially in the early stages of decay or when dealing with less aggressive fungi. Furthermore, data on the low mass loss and hyphal abundance of *E. parasitica* is consistent with observations of the slow progress of this species and wood decay in the field. The average annual growth of the fungus in length is 1–2 cm [43]. The extension of the canker is faster in the longitudinal direction than in the transverse direction [44]. Ogris, et al. [45] stated that infected older maples can live and grow with fungus for many decades. But due to the progress of fungal activity, wood decay and loss of mechanical properties, it is very common for older infected trees to snap at the canker and fall over [13,46].

Measured cell wall thickness correlated well with the calculated mass loss of the wooden mini blocks. Successful colonization and effective degradation of woody tissue resulted in higher mass loss. This is most evident in samples exposed to TV. Although *E. parasitica* isolates caused about four times smaller mass loss of samples compared to TV and TVD, we should not disregard their capability of degrading maple wood. Because *E. parasitica* usually infects the lower portion of the trunk, which is the thickest and most valuable part of the tree, any damage is undesirable, as even incipient decay can result in significant loss of mechanical properties [36].

The natural durability of wood to fungal activity could be indicated by wood density, one of the most important wood quality characteristics. There are studies which confirmed that lower density results in higher susceptibility to fungal decay [40,47]. This could also be the case in our study. In addition, we assume that the early wood of maples is also more susceptible to fungal colonization and decay. Early wood showed slightly thinner fiber and vessel walls, which correspond to slightly lower densities in comparison to other parts of the annual wood increment, i.e., transition wood and late wood.

Based on the results of calculated wood density and moisture content, we assumed that *A. campestre* wood had somewhat different characteristics from the other two maple species used in this experiment. The wood of *A. campestre* was significantly denser and had higher average moisture content. However, the correlation between moisture content and wood density was not significant. The higher density of *A. campestre* wood, in most cases, did not correspond to lower susceptibility to fungal decay and consequently to lower mass loss, as would be expected. This suggests that the relationship between fungal and tree species may also play an important role in the characteristics of wood decay.

Wood discoloration and decay in trees is usually a consequence of bacterial and fungal colonization of branch stubs, which expose the wood of living trees [48]. Decay characteristics and progress can be explained by the CODIT (compartmentalization of decay in trees) model developed by Shigo and Marx [49]. Green, et al. [50] studied compartmentalization of discolored and decayed wood initiated with the loss of branches in young *A. rubrum* L. They distinguished two basic types of dead branch stubs, some with a clearly defined green-colored boundary, which separated the discolored and decayed wood of the stub from sapwood inside the trunk, and a second type of stub that lacked

this boundary and where discolored wood extended into the trunk. Green, Shortle, and Shigo [50] found that failure in the formation or destruction of the boundary tissues may facilitate the spread of microorganisms, discoloration and decay into the trunk. Formation of this barrier is also of great interest in *E. parasitica* infections. *Eutypella parasitica* infects the trunk through a branch stub or bark wound and triggers more or less successful compartmentalization. When this is not the case, the fungus progresses into the sapwood and causes characteristic wood discoloration and decay. However, this has yet to be studied.

5. Conclusions

Fifteen weeks of exposure to ten different fungal species resulted in notable mass loss of *Acer pseudoplatanus*, *A. platanoides*, and *A. campestre* wood samples, according to the modified decay test of EN 113. The fungal species used in the experiment had different decay potentials. As expected, *Trametes versicolor* and *Gloeophyllum trabeum* were the most efficient species in the colonization and decay of the maple samples. Other fungi (*Diaporthe* sp., *Eutypa* sp., *Eu. maura*, *E. parasitica*, *Fusarium* sp., *Neocucurbitaria acerina*, *Neonectria faginata*, and *N. punicea*) were less effective. Any visually evident staining or discoloration of wood samples associated with their activity was not noticed. There was a significant correlation between mass loss and moisture content. Higher mass loss corresponded to higher moisture content of wood samples after incubation. Colonization and decay of samples were additionally confirmed by light and scanning electron microscopy, which revealed abundant hyphal growth in the vessels and sometimes significant degradation of cell walls. Changes in cell walls were confirmed by measurements of fiber and vessel wall thickness in the early wood, transition wood, and late wood. The results revealed significantly thinner cell walls in samples which experienced higher mass loss and degradation. Although *E. parasitica* caused significantly lower mass loss of samples compared to other more effective species, we should not disregard its capability of degrading maple wood, particularly as we were not able to fully mimic the natural microclimate in the trees in the laboratory. Based on the results of staining, we assumed that *E. parasitica* was able to degrade lignin and could therefore be considered as a white rot fungus.

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3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

3.1.1 Združbe gliv, ki se pojavljajo na odmrlih vejah gorskega javorja

Kot prvi del raziskave in osnova za nadaljevanje dela je služil naš prvi cilj: *Določiti najpogostejše vrste gliv v lesu odmrlih vej gorskega javorja na petih lokacijah v okolici Ljubljane.* S pomočjo Zavoda za gozdove Slovenije smo poiskali primerne lokacije z zadostnim številom mladih gorskih javorjev. Pri izbiri lokacij nas v osnovi ni zanimala prisotnost *E. parasitica* v sestoju ali bližnji okolici, temveč zgolj najpogostejše vrste gliv, ki se pojavljajo v lesu odmrlih vej gorskega javorja. Združba gliv bi namreč lahko neposredno ali posredno vplivala na okužbe in rast *E. parasitica*. Z izolacijami iz lesa odmrlih vej gorskega javorja smo zabeležili pester izbor glivnih vrst. Najpogosteje izolirane vrste so bile *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 in *E. parasitica*. Opozoriti velja, da je dejanskih vrst v lesu odmrlih vej gorskega javorja zagotovo precej več in da so v vzorcih zagotovo tudi druge, neprepoznane, vrste (Wu in sod., 2019). Zaradi časovne in finančne omejitve smo se omejili le na določitev najpogosteje izoliranih morfotipov, tj. vrst, ki so bile identičnega makromorfološkega videza. Pri razvrščanju kultur v morfotipe smo bili pozorni na obliko, privzdignjenost, rob in barvo kulture ter morebitne posebnosti. Na tak način smo določili le vrste z najhitrejšo rastjo in tiste, ki so sposobne rasti v uporabljenih razmerah izolacije in inkubacije. Določitev morfotipov na podlagi makromorfoloških lastnosti kolonij je sicer časovno hitrejša, kot je razločevanje z mikroskopijo ali drugimi tehnikami, vendar pa ima tudi svoje slabosti: obstaja namreč tveganje, da ne zaznamo določenih, t. i. kriptičnih vrst. To so vrste, ki imajo identične morfološke značilnosti, vendar se razlikujejo v genetskem zapisu (Balasundaram in sod., 2015).

Z večanjem debeline odmrlih vej gorskega javorja se je v naši raziskavi večalo tudi povprečno število izoliranih vrst. Ker bi bile debelejše veje lahko tudi starejše, sledi logičen zaključek o večjem številu glivnih vrst na starejših vejah. Vendar pa v sklopu tega cilja nismo ugotavljali starosti in raziskovali vpliva starosti vej na vrstno pestrost gliv. Predvidevamo, da v naši raziskavi starost ni "moteč" dejavnik, saj so bila vsa drevesa približno enake starosti (do deset let) in tako se tudi debelina oz. starost vej ni značilno spreminjala. To potrjuje tudi dejstvo, da so bile razlike v povprečnem številu vrst med različnimi debelinskimi razredi vej neznačilne. Smo pa z izračunom Jaccardovega indeksa podobnosti (C_J) med glivnimi združbami različnih debelinskih razredov vej odkrili relativno visoko povprečno vrednost – 0,80.

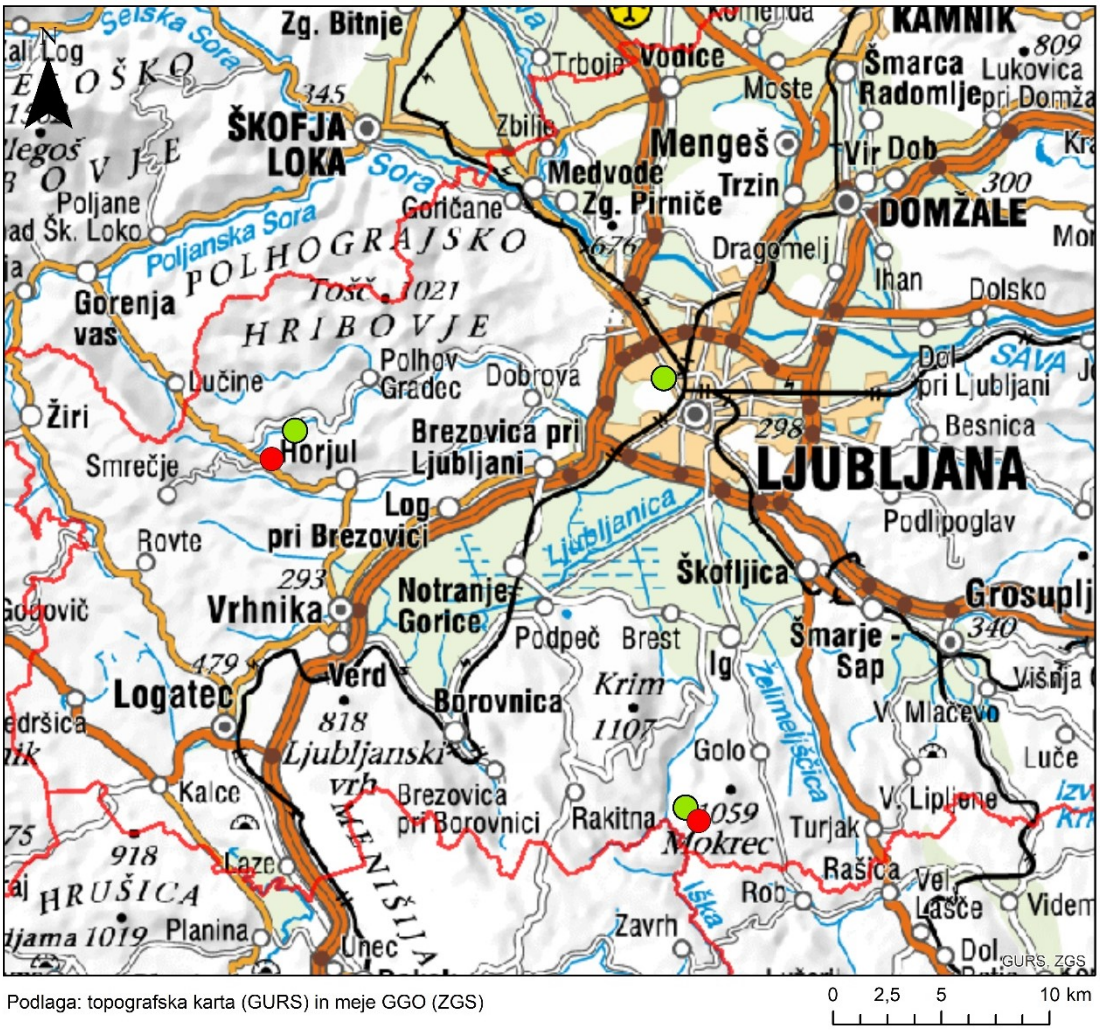
V raziskavi smo med 91 morfološko izbranimi morfotipi z molekularnimi metodami določili 58 taksonov. Lokacije vzorčenj so si bile relativno blizu (največja zračna razdalja 26 km), kar kaže na veliko pestrost glivnih vrst. V literaturi smo iskali razloge za relativno veliko

pestrost v številu vrst v primerjavi z drugimi raziskavami (Butin in Kowalski, 1986; Chlebicki, 1988; Kowalski in Kehr, 1992; Unterseher in sod., 2005; Johnova, 2009) in ugotovili, da gre najverjetneje za kombinacijo ugodnejše geografske lokacije, podnebnih značilnosti, starosti in velikosti vzorcev, metode sterilizacije in različnih mest izolacije (Danti in sod., 2002; Tedersoo in sod., 2014). Med prepoznanimi glivami so se za dominantne vrste v odmrlih vejah gorskega javorja izkazale *Eu. maura*, *Eutypa* sp. 2, *F. avenaceum*, *N. acerina* in *E. parasitica*. Samo *Eu. maura* se pojavlja tudi v drugih raziskavah, preostale vrste pa so specifične za naše rezultate.

Iz treh vzorcev v naši raziskavi so bile izolacije neuspešne, sicer pa smo določili širok spekter glivnih vrst. Večinoma so prepoznane vrste spadale med zaprtotrosnice (Ascomycota) in prostotrosnice (Basidiomycota) (Butin in Kowalski, 1986; Kowalski in Kehr, 1992). V več kot 20 % vzorcev sta se pojavili le dve vrsti (*Eu. maura* in *F. avenaceum*). Nekoliko neznačilna, vendar razumljiva je bila izolacija *Eutypa* sp. 3, *Eu. maura*, *Daldinia* sp. Ces. & De Not. in *Dendryphion europaeum* Crous & R.K. Schumach. iz kontrolnih vzorcev. Domnevamo, da je razlog v njihovi endofitski življenjski strategiji. Večina gliv, ki smo jih določili, je sicer znanih saprofitov. Med najpogosteje prepoznanimi rodovi je bil rod *Eutypa* spp. z vrstama *Eu. maura* in *Eu. lata* Tul. & C. Tul. *Eutypa maura* je precej razširjena in pogosto dominantna vrsta odmrlih poganjkov in vej. Njena stroma lahko hitro preraste površino vej in drugim sekundarnim glivam onemogoči uspešno naselitev substrata (Unterseher in Tal, 2006). *Eutypa lata* je svetovno razširjen patogen različnih lesnatih rastlin (Trouillas in Gubler, 2010; Wenneker in sod., 2011; Travadon in sod., 2012). Najbolj znana je kap vinske trte oz. evtipoza, ki jo povzroča na *Vitis vinifera* L. (Rolshausen in sod., 2008; Trouillas in Gubler, 2010; Lešnik in Mešl, 2021). Obstaja pa tudi posebna varieteta *Eu. lata* na lesu in skorji maklena ter gorskega javorja, ki jo je opisal Rappaz (1987) v Franciji in Švici. Med pogosto izoliranimi vrstami je bila tudi *F. avenaceum*, ki spada med najpogostejše vrste iz rodu *Fusarium* v zmernih podnebnih razmerah, in povzroča padavico kalčkov lesnatih rastlin ter trohnobo korenin, stebel in plodov žit ter stročnic (Maček, 2008; Peters in sod., 2008; Sørensen in sod., 2009). Omenimo lahko še eno izmed petih najpogosteje izoliranih vrst – *N. acerina*. To je vrsta, ki so jo opisali leta 2017 in živi kot endofit ali saprofit na maklenu, našli pa so jo na odmrlih maklenovih poganjkih v Italiji. *Neocucurbitaria acerina* so predlagali kot mogočo spolno obliko *Pyrenochaeta quercina* Kabát & Bubák (Wanasinghe in sod., 2017).

Med petimi najpogosteje izoliranimi vrstami gliv iz lesa odmrlih vej gorskega javorja je bila tudi povzročiteljica javorovega raka, *E. parasitica*. Zanimiv in po svoje presenetljiv rezultat so izolacije *E. parasitica* iz vzorcev z vseh lokacij, čeprav na dveh (Mokrc in Samotorica) pri inventuri nismo opazili razvitih rakov (Slika 6). Na lokaciji Rožnik je bilo kar 45 % vzorčenih dreves okuženih z *E. parasitica*, vendar vsa drevesa niso kazala znakov okužbe. V povprečju je bilo na posamezni lokaciji okuženih 19 % vzorčenih dreves, kar se sklada z rezultati drugih raziskovalcev, ki navajajo povprečno 3–5 % oz. do 30 % pojavnost bolezni

v sestojih (Gross, 1984b; Ogris in sod., 2009). Glede na rezultate izolacij in inventure je *E. parasitica* očitno bolj razširjena, kot je bilo znano doslej. Verjetno je bila bolezen enostavno spregledana, saj ni sistematičnega monitoringa in je mlade okužbe težko opaziti (Jurc in sod., 2016). Morda gre za sposobnost glive, da povzroči asimptomatske okužbe ali zmožnost saprofitskega načina prehranjevanja ali pa za katere druge neznane razloge.



Slika 6: Lokacije inventure in vzorčenja (zelena barva označuje prisotnost javorovega raka, rdeča barva pa odsotnost javorovega raka).

Figure 6: Monitoring and sampling sites (green indicates the presence of Eutypella canker, red indicates the absence of Eutypella canker).

S statističnimi izračuni nismo ugotovili značilnih razlik v pestrosti vrst in glivnih združbah med vzorci z *E. parasitica* ali brez nje. Nobena izmed najpogosteje izoliranih vrst ni bila strogo povezana s pojavnostjo *E. parasitica*. Ugotovili smo pogostejše izolacije *E. parasitica* iz razbarvanega lesa v deblu kot iz zunanjega dela odmrle veje. Predvidevamo, da je v zunanjem delu odmrle veje velika konkurenca, zaradi katere *E. parasitica* hitro napreduje v les debla in jo v zunanjem delu veje nadomestijo druge, konkurenčnejše vrste. Razlike v

združbi gliv med različnimi mesti izolacije niso nič nenavadnega, saj gre za drugačen substrat in razmere, v katerih vrste rastejo in se razvijajo.

Rezultati naše raziskave kažejo na izjemno veliko pestrost prepoznanih vrst v lesu odmrlih vej gorskega javorja. Shannonov diverzitetni indeks (H´) je znašal 3,01–3,30 ($p = 0,076$) med različnimi lokacijami vzorčenja, 3,51–3,59 ($p = 0,212$) med različnimi mesti izolacije in 3,56–3,71 ($p = 0,822$) med različnimi debelinskimi razredi vej. Po navadi so vrednosti H´ od 1,5 do 3,5 in le redko presežejo 4,0 (Magurran, 2004). Vrednosti H´ pri drugih drevesnih vrstah v tujih študijah so precej nižje od vrednosti, ki smo jih izračunali v naši raziskavi: 0,21–0,80 v tkivih *Quercus robur* L. in *Q. cerris* L. (Gennaro in sod., 2003) ter 1,28–2,11 pri *Ulmus macrocarpa* Hance, *Q. liaotungensis* Koidz. in *Betula platyphylla* Sukaczev (Sun in sod., 2012). Podobnost združb gliv med različnimi lokacijami vzorčenja je bila relativno majhna (0,59) v primerjavi s Kowalski in Kehr (1992), ki sta izračunala vrednosti 0,65–0,92 med živimi in odmrlimi debli ter poganjki *Fraxinus excelsior* L.

Prepoznane glivne združbe so se značilno razlikovale med lokacijami vzorčenja, med mesti izolacije v vejah in med debelinskimi razredi vej ($p < 0,003$). Na sestavo, številčnost, in razporeditev gliv v lesu odmrlih vej gorskega javorja lahko vplivajo številni dejavniki (Kowalski in Kehr, 1992; Danti in sod., 2002; Gennaro in sod., 2003; Unterseher in sod., 2005; Unterseher in Tal, 2006; Kowalski in sod., 2016; Hanácková in sod., 2017):

- gostitelj (gostiteljevo splošno zdravstveno stanje, stopnja razkroja vzorcev, starost dreves in vej, debelina vej),
- okolje (temperatura, padavine, značilnosti tal, razpoložljivost vode, drevesna sestava, rastlinska združba),
- značilnosti vzorčenja, izolacije in inkubacije (čas vzorčenja, metoda izolacije, posplošene inkubacijske razmere).

Po predvidevanjih *E. parasitica* izhaja iz Severne Amerike in so jo v Evropo prinesli s sadikami okrasnega drevja (Ogris in sod., 2006). Da bi ugotovili natančnejši izvor vrste in določene populacijske značilnosti, bi bilo v prihodnje smiselno razmišljati o izvedbi populacijskih študij *E. parasitica*. Na to temo že poteka študija v sklopu magistrskega dela študenta biotehnologije (Marinč, neobjavljeno), katerega preliminarni rezultati kažejo na razlike med evropsko in severno ameriško populacijo *E. parasitica*.

Z našo prvo raziskavo smo določili najpogostejše vrste gliv v odmrlih vejah gorskega javorja na petih lokacijah v okolici Ljubljane, kar nam je služilo kot osnova za nadaljnje raziskovanje medvrstnih vplivov in hitrosti razkroja lesa, o čemer razpravljamo v nadaljevanju.

3.1.2 Konkurenčnost gliv in morebiten antagonizem

Vrste, ki smo jih izolirali in določili v naši raziskavi, so večinoma znani saprofiti listavcev. Ker se v našem delu usmerjamo na *E. parasitica,* je še posebno zanimiva prepoznava hiperparazitov *Eutypella* spp. in *Eutypa* spp., ki bi jih lahko potencialno uporabili za biološko kontrolo. S tega vidika je treba omeniti določitev naslednjih vrst: *Bloxamia* sp., *Cosmospora* sp. in *Pseudocosmospora rogersonii*. *Pseudocosmospora rogersonii* je v ZDA prepoznana kot mikoparazit *Eutypella* spp. (Herrera in sod., 2013). Podobno velja tudi za *Cosmospora* spp., ki parazitira vrste iz reda Xylariales (Herrera, 2014). *Bloxamia truncata* pa se pojavlja na delno razkrojeni stromi *Eutypella* spp., zato so raziskovalci predlagali študijo njene potencialno mikoparazitske aktivnosti (Glawe, 1984).

Z obdelavo podatkov najpogosteje izoliranih vrst nismo ugotovili značilnih razlik v vrstni pestrosti in združbi gliv med vzorci z *E. parasitica* in brez nje. To lahko kaže na dejstvo, da najpogosteje izolirane vrste ne zavirajo ali spodbujajo njene rasti. Da bi podrobneje proučili njihov vpliv, smo določili naslednji raziskovalni cilj: *Izmeriti vpliv desetih najpogostejših vrst gliv v lesu odmrlih vej gorskega javorja na rast glive E. parasitica v čisti kulturi in ugotoviti njihov morebitni antagonizem.* Predvidevali smo, da je gliva, ki povzroča javorov rak, manj konkurenčna drugim glivam, ki se prav tako pojavljajo v odmrlih vejah, saj bi bil v nasprotnem primeru javorov rak verjetno pogostejši. Zaviralni učinek desetih najpogosteje izoliranih vrst gliv v lesu odmrlih vej gorskega javorja na izolat *E. parasitica* smo raziskali s testom dvojnih kultur.

Izzivalni izolati, ki smo jih uporabili v dvojnih kulturah, so se večinoma izkazali za šibke antagoniste (vrednosti antagonističnega indeksa; AI < 10), razen *Eutypa* sp., *Eu. maura*, *Neonectria* sp. in *P. incarnata* s srednjimi vrednostmi AI (10–15). Glede na kategorije, ki so jih oblikovali Badalyan in sod. (2002), nismo odkrili visoko aktivnih antagonistov (AI > 15) *E. parasitica*. Zaradi v določenih primerih zelo podobnega makroskopskega videza kultur je bilo težko določiti tip interakcije in izbrati izolat, ki je preraščal drugega. Posebno nehvaležno je bilo razlikovanje med ustavitvijo rasti po stiku micelijev (tip A) in ustavitvijo rasti na razdalji brez stika micelijev (tip B). Pri določanju tipa interakcije smo sledili klasifikaciji, ki so jo razvili Badalyan in sod. (2002) in poleg že omenjenega tipa A in B razlikovali še tip C. V ta sklop so bile uvrščene dvojne kulture, kjer smo določili, da je eden izmed izolatov delno ali v celoti prerasel drugega (podtipi C_{A1}, C_{A2}, C_{B1} in C_{B2}). *Eutypa* sp., *Eu. maura*, *Neonectria* sp. in *P. incarnata* so bili edini izolati, ki so prerasli *E. parasitica*. V primeru dvojnih kultur *E. parasitica* in *Diaporthe* sp. ter *N. acerina* smo opazili različne tipe interakcij znotraj istih obravnavanj, o čemer poročajo tudi drugi raziskovalci (Rayner in sod., 1994; Kusari in sod., 2013; Hanácková in sod., 2017; Rezgui in sod., 2018).

Eutypella parasitica ni prerasla nobenega od izzivalnih izolatov, kar nakazuje na njeno relativno neagresivnost in šibko tekmovalnost v primerjavi z izzivalnimi izolati. Slednje je

nakazala že predhodna raziskava najpogosteje izoliranih vrst gliv v lesu odmrlih vej gorskega javorja. Ugotovili smo namreč pogostejše izolacije *E. parasitica* iz debla, kar je posledica velike konkurence v zunanjem delu odmrle veje, zaradi česar gliva hitro napreduje v les debla.

Reizolacije iz dvojnih kultur niso bile vedno uspešne (primer dvojne kulture z *F. avenaceum* in *P. incarnata*). Če smo iz interakcijske cone reizolirali obe vrsti, smo predvidevali manjšo konkurenčnost izzivalnega izolata. Ko je uspela samo reizolacija izzivalnega izolata, pa smo predvidevali, da je bila *E. parasitica* v interakcijski coni premagana in sklepali na večjo tekmovalno uspešnost izzivalnega izolata. V primeru večje uspešnosti smo beležili višje vrednosti uspešnosti reizolacij (s) in obratno. Negativne vrednosti uspešnosti reizolacij smo izračunali v primeru dvojnih kultur z *Diaporthe* sp., *N. acerina* in *Pe. irregularis*, kar kaže na njihov manjši tekmovalni uspeh. Pri reizolacijah iz interakcijske cone tipa A smo večinoma uspešno reizolirali oba testna izolata, občasno celo iz istega koščka. Podoben rezultat je v literaturi (Koukol in sod., 2006) omenjen tudi za reizolacije iz interakcijske cone tipa B. V naši raziskavi smo sicer le v 20 % primerov reizolirali oba izolata, v preostalih primerih pa je bila uspešna samo reizolacija *E. parasitica*. Z reizolacijami smo ugotovili, da je ostal micelij *E. parasitica* v interakcijski coni večinoma aktiven (tudi v primeru delnega preraščanja s *P. incarnata*), razen v dvojnih kulturah z *Eutypa* sp., *F. avenaceum* in *Neonectria* sp., kjer reizolacije *E. parasitica* niso bile uspešne. Iz tega lahko sklepamo, da je *E. parasitica* šibek tekmec, ki vlaga manj energije v neposredno micelijsko tekmovanje z omenjenimi tremi izolati (Koukol in sod., 2006).

Če na kratko povzamemo naše ugotovitve glede *in vitro* interakcij, lahko na podlagi vrednosti antagonističnega indeksa (AI) zaključimo, da so *Eutypa* sp., *Eu. maura*, *Neonectria* sp. in *P. incarnata* značilno negativno vplivale na rast *E. parasitica* v dvojni kulturi. Rezultati se dobro ujemajo tudi s pričakovano visoko uspešnostjo reizolacij izzivalnih izolatov iz interakcijske cone. Na podlagi uspešnosti reizolacij (s) sta se *Eutypa* sp. in *Neonectria* sp. izkazali za dobra inhibitorja rasti *E. parasitica*. Reizolacije *E. parasitica* iz interakcijske cone so bile neuspešne, kar lahko pomeni, da sta jo izzivalna izolata premagala. Podoben učinek v dvojnih kulturah sta imeli tudi *Eu. maura* in *P. incarnata*, kjer smo izračunali visoke vrednosti AI in s. V interakcijskih conah omenjenih dvojnih kultur smo opazili nekoliko makroskopsko spremenjen micelij, kar je lahko prvi znak premoči in nadomestitve testnega izolata *E. parasitica*. Na drugi strani sta imeli *N. acerina* in *Diaporthe* sp. relativno nizek in neznačilen AI ter s tem povezane tudi nižje vrednosti s, kar kaže na večji tekmovalni uspeh *E. parasitica* v primeru omenjenih interakcij *in vitro*. Dvojne kulture *E. parasitica* in *F. avenaceum* z nizkim inhibitornim učinkom in nizko uspešnostjo reizolacij izzivalnega izolata so dober pokazatelj kompleksnosti interspecifičnih interakcij, ki lahko občasno postrežejo z nenavadnimi in nepričakovanimi rezultati (Yuen in sod., 1999).

Izolati gliv *Eutypa* sp., *Eu. maura*, *Neonectria* sp. in *P. incarnata* so se torej izkazali za najbolj obetavne za uporabo v sklopu biološke kontrole *E. parasitica*, vendar pa pri tem ne smemo pozabiti na potrebo po dodatnih raziskavah. Pri interpretaciji in posploševanju pridobljenih rezultatov moramo biti izjemno previdni. V prihodnje bi bilo priporočljivo zasnovati dodatne teste z več ponovitvami in s širšim naborom izolatov *E. parasitica*. V poskus bi lahko kot izzivalne izolate vključili tudi: *Bloxamia* sp., *Cosmospora* sp. in *Pseudocosmospora rogersonii*, ki jih v literaturi omenjajo kot potencialne antagoniste. Dodatno bi lahko raziskali tudi sekundarne metabolite in njihov vpliv na antagonistično aktivnost. Med interspecifičnimi interakcijami namreč nastajajo in sproščajo številne bioaktivne snovi (Yuen in sod., 1999; Prior in sod., 2017), ki lahko zavirajo rast, vplivajo na pigmentacijo v interakcijski coni, barvo kolonije ipd. (Asthana in Shearer, 1990; White in Boddy, 1992; Yuen in sod., 1999; Badalyan in sod., 2002, 2004; Evans in sod., 2008). Za vrste, ki proizvajajo malo sekundarnih metabolitov, velja, da so manj tekmovalne, ne izrabljajo dobro prostora in imajo manjši antagonistični potencial (Yuen in sod., 1999). Načeloma velja, da klimaksne vrste proizvajajo več antagonističnih substanc, so zato bolj tekmovalne in uspešneje zavirajo rast vrst, ki se pojavijo v začetku sukcesije (Yuen in sod., 1999). Smiselno bi bilo raziskati tudi interakcije v naravnem okolju, kar pa že presega cilj te raziskave. V laboratoriju namreč ni mogoče zagotoviti razmer, enakovrednih tistim v naravi – inokulacijski potencial, uspešnost kalitve, mikroklima, celotna združba gliv, naraven substrat ipd. (Yuen in sod., 1999; Boddy, 2000). So pa zagotovo testi v laboratoriju prvi korak k razumevanju interakcij in iskanju potencialno učinkovitih strategij biološke kontrole (Yuen in sod., 1999).

Raziskave biološke kontrole bolezni dreves oz. lesnatih rastlin so relativno redke v primerjavi s tistimi na zelnatih, enoletnih rastlinah. Kompleksnost razvitja učinkovitih metod biološke kontrole je posledica velike biomase, zapletene anatomije, dolgoživosti in posebnosti gospodarjenja z gozdovi (Cazorla in Mercado-Blanco, 2016). Kljub omejitvam so raziskovalci razvili nekaj praktičnih ukrepov biokontrole, ki se v večini primerov osredotočajo na mlajša drevesa, po navadi v fazi sadik v drevesničarski proizvodnji (Cazorla in Mercado-Blanco, 2016). Vprašanje, ki se pojavi na tem mestu, je, ali je mogoče tovrstne ukrepe učinkovito uporabiti tudi na odraslih osebkih, v naravnih razmerah in na velikih površinah. Dodaten pomislek je tudi ekonomičnost takih ukrepov (Cazorla in Mercado-Blanco, 2016). Hardoim in sod. (2015) kot zanimivo in uporabno možnost biološke kontrole navajajo uporabo endofitnih organizmov. Njihova prilagodljivost in vzdržljivost sta najpomembnejši lastnosti, ki omogočata potencialno uporabo v okviru vzdrževanja zdravja gozdov (Pautasso in sod., 2015). V gozdni fitopatologiji že obstaja nekaj uspešnih zgodb biološke kontrole, npr. uporaba *Verticillium alboatrum* Reinke & Berthold pri holandski brestovi bolezni (*Ophiostoma ulmi* (Buisman) Nannf. in *Ophiostoma novo-ulmi* Brasier), uporaba *Phlebiopsis gigantea* (Fr.) Jülich pri rdeči trohnobi korenin (*Heterobasidion* sp. Bref.), hipovirulenca kostanjevega raka (*Cryphonectria parasitica* (Murrill) M.E. Barr) ipd. (Cazorla in Mercado-Blanco, 2016).

V slovenskih gozdovih je uporaba fitofarmacevtskih sredstev dovoljena le v določenih primerih in pod določenimi pogoji, ki jih določi Zavod za gozdove Slovenije (Pravilnik o varstvu gozdov, 2009). Znana sta primera uporabe sredstva AQ-10 na osnovi hiperparazitske glive *Ampelomyces quisqualis* Ces. proti hrastovi pepelovki (*Erysiphe alphitoides* (Griffon & Maubl.) U. Braun & S. Takam.) (MKGP, 2020) in vnos tujerodne parazitske osice *Torymus sinensis* Kamijo za zatiranje kostanjeve šiškarice (*Dryocosmus kuriphilus* Yasumatsu) (Rot in sod., 2017).

3.1.3 Obarvanje in razkroj lesa

Biološka kontrola *E. parasitica* bi bila odličen način, da bi se izognili ali vsaj čim bolj zmanjšali negativne učinke patogena. Okužbe z glivo *E. parasitica* namreč povzročijo veliko tehnično in ekonomsko razvrednotenje najvrednejših sortimentov (Gross, 1984b; Martinez in sod., 2003). Da bi podrobneje proučili razkroj lesa, smo si postavili cilj: *Izmeriti izgubo lesne mase treh vrst javorjev (Acer pseudoplatanus, A. platanoides, A. campestre) zaradi delovanja glive E. parasitica po metodi mini-blok, ki temelji na standardu SIST EN 113:2002, in primerjati rezultate z izolati gliv Trametes vericolor, Gloeophyllum trabeum in s petimi najpogostejšimi glivami, ki povzročajo trohnobo v javorjevih odmrlih vejah.* Predvidevali smo, da bo razkroj lesa javorjev zaradi *E. parasitica* relativno počasna v primerjavi z drugimi trohnobnimi glivami, ki smo jih vključili v poskus.

Vzorce lesa gorskega, ostrolistnega in poljskega javorja smo za 15 tednov izpostavili izolatom gliv: *Diaporthe* sp., *Eutypa* sp., *Eu. maura*, *E. parasitica* (4x), *Fusarium* sp., *Gloeophyllum trabeum* (Pers.) Murrill (2x), *N. acerina*, *Neonectria faginata* (M.L. Lohman, A.M.J. Watson & Ayers) Castl. & Rossman, *Ne. punicea* (J.C. Schmidt) Castl. & Rossman in *Trametes versicolor* (L.) Lloyd (dva izolata). Vsi glivni izolati so povzročili večje izgube lesne mase od kontrol. V nekaterih primerih je bila izračunana izguba mase manjša od 3 %, kar glede na standard SIST EN 113:2002 (CEN, 2002) velja za neznačilno.

Po pričakovanjih sta bili *T. versicolor* in *G. trabeum* najučinkovitejši vrsti pri naselitvi in razkroju vzorcev lesa. Obe vrsti sta znani, da hitro in uspešno naseljujeta odmrl substrat ter povzročata učinkovit razkroj različnih vrst lesa po svetu (Robinson in sod., 2007; Humar in sod., 2018). *Trametes versicolor* oz. pisana ploskocevka je zelo razširjena prostotrosnica, ki se razvija v odmrli beljavi listavcev in povzroča belo trohnobo lesa. Je saprofit vej in debel, posekanega lesa, drogov in panjev. Trosnjaki so tanki, kožasti, trdi, široki do 5 cm in enoletni. Po zgornji strani so gostodlakavi s conami od bele, rjave, sive do črne barve; spodaj so rumeni z drobnimi okroglimi porami (Maček, 2008). *Gloeophyllum trabeum* oz. navadna tramovka spada med najbolj kozmopolitanske vrste in povzroča rjavo trohnobo lesa. Najpogosteje se pojavlja na skladiščih, vrtnem pohištvu, ograjah, telekomunikacijskih drogovih, ostrešjih, mostovih, lesenih plovilih in oknih. Posebno pogosto razkraja tehnični les, ki se občasno navlaži. Pojavlja se na lesu listavcev in iglavcev. Trosnjaki so žilavi in

prožni, enoletni, različnih oblik, pogosto se pojavljajo v vrstah. Na zgornji strani so dobro vidne rjave koncentrične prirastne plasti, površina je rahlo razbrazdana; spodaj je gosta lamelasta trosovnica oker do rjave barve (Humar, 2008).

Povprečna izguba mase zaradi delovanja izolatov *T. versicolor* v vzorcih gorskega in ostrolistnega javorja je bila v naši študiji skoraj dvakrat večja od rezultatov tujih študij (Kielmann in sod., 2014; Reinprecht in sod., 2016). Omenjene razlike lahko pripišemo krajšemu oz. daljšemu trajanju poskusa, različnemu izvoru, vitalnosti in moči izolatov, velikostim vzorcev, inkubacijskim razmeram, gojišču ipd. Naši rezultati kažejo na relativno veliko občutljivost lesa javorjev za delovanje omenjene glive, povzročiteljice bele trohnobe. Relativna razlika v povprečni izgubi mase v primeru izolatov *G. trabeum* DSMZ (nemška zbirka mikroorganizmov in celičnih kultur; nem. *Deutsche Sammlung von Mikroorganismen und Zellkulturen*) in *G. trabeum* ZIM (Zbirka industrijskih mikroorganizmov Katedre za biotehnologijo na Biotehniški fakulteti) je bila precej velika (beležili smo 2,8 × oz. 1,2 × večje izgube pri izolatih *G. trabeum* DSMZ kot pri *G. trabeum* ZIM). To kaže na možnost neuspešne inokulacije v primeru vzorcev gorskega javorja in delno tudi ostrolistnega. Naš sum smo potrdili tudi z neznačilno razliko v povprečni masi absolutno suhih vzorcev lesa gorskega javorja pred izpostavitvijo izolatu *G. trabeum* ZIM in potem ter neznačilne razlike v izgubi mase vzorcev gorskega in ostrolistnega javorja v primeru izpostavitve *G. trabeum* ZIM in kontrolnih vzorcev. Na podlagi podatkov o vsebnosti vode na koncu poskusa ugotavljamo, da je bila gliva vsekakor aktivna, vendar je bila moč njenega razkroja precej pod pričakovanji.

Rezultati izgube mase zaradi delovanja izolatov *E. parasitica* so primerljivi poročilom, kot jih je navajal French (1967) o povprečnih izgubah mase vzorcev rdečega, sladkornega in srebrnega javorja. Ugotovil je nekoliko večje izgube mase v beljavi kot jedrovini. Takih razlik v sklopu našega poskusa nismo preverjali. V nasprotju z našimi ugotovitvami French (1967) ni poročal o značilnih razlikah v povprečni izgubi mase med različnimi izolati *E. parasitica*. Tudi Pildain in sod. (2005) so ugotavljali, kakšen je razkroj lesa *Po. deltoides* s štirimi različnimi izolati *Eutypella* spp. in izračunali v povprečju za 1,7- do 7,0-krat višje izgube mase od naših rezultatov. Worrall in sod. (1997) pa so beljavo *B. alleghaniensis* in *Pi. taeda* izpostavili delovanju *E. parasitica* in zabeležili primerljive vrednosti (5,6 % pri *B. alleghaniensis* in 2,3 % pri *Pi. taeda*). Pri tovrstnih primerjavah izgub mase lesa moramo opozoriti na razlike v uporabljenih metodologijah in biti previdni pri oblikovanju hitrih zaključkov.

Preostali izolati, ki smo jih uporabili v poskusu, so povzročili relativno majhne izgube mase (0,45–13,07 %) in smo jih v poskus vključili zaradi povezave s predhodnima raziskavama ter morebitnih zanimivih rezultatov za nadaljnje raziskave.

Razmerje med izgubo mase in vsebnostjo vode kaže na linearno pozitivno povezavo, kar ugotavljajo tudi drugi raziskovalci (Meyer in sod., 2016; Zelinka in sod., 2020). Izračunana vsebnost vode naših vzorcev je bila relativno velika. Ker vzorci lesa niso bili v neposrednem stiku z gojiščem (to je preprečevala uporaba plastične mrežice), lahko sklepamo, da je velika vsebnost vode posledica delovanja gliv. V teoriji se namreč ob razkroju enega grama celuloze sprosti 0,555 g vode (Griffin, 1977). Težko je reči, ali je manjša izguba mase posledica manjše vsebnosti vode ali pa je manjša vsebnost vode posledica manjše izgube mase (Zelinka in sod., 2020). V ta namen so raziskovalci predlagali izračun dodatne spremenljivke, t. i. razlike v vsebnosti vode. Slednja naj bi predstavljala dodatno vodo, ki jo proizvede sama gliva (Zelinka in sod., 2020). Naši rezultati so pokazali povezavo med uspešno glivno presnovo, kar se kaže z večjimi izgubami mase lesa in večjo končno vsebnostjo vode. Še posebno dobro je ta povezava opazna pri podatkih, ki se nanašajo na delovanje izolatov *T. versicolor* in *G. trabeum*. Negativne vrednosti razlik v vsebnosti vode pri vzorcih ostrolistnega javorja, ki smo jih izpostavili *Eu. maura*, ne znamo pojasniti. Pregled literature je sicer pokazal, da se to dogaja tudi v drugih raziskavah, vendar avtorji prav tako ne navajajo razlogov (Peterson in Cowling, 1973; Zelinka in sod., 2020).

Neznačilne razlike v povprečni vsebnosti vode med kontrolnimi vzorci in vzorci, izpostavljenimi določenim glivnim izolatom, kažejo, da izolati niso proizvedli velikih količin vode, kar se sklada z njihovo manjšo sposobnostjo razkroja lesa.

V nadaljevanju smo zbirali dodatne informacije o naselitvi in razkroju lesa s svetlobno (LM) in vrstično elektronsko mikroskopijo (SEM). Testirane glive (*T. versicolor*, dva izolata *E. parasitica*) so se razraščale v trahejah, ki so najširše celice v javorjevem lesu in so nekakšna glivna avtocesta (Humar in sod., 2008). Z mikroskopijo smo potrdili, da je izguba mase resnično posledica delovanja gliv. V naši raziskavi se je kot najuspešnejša gliva za naselitev in razkroj lesa gorskega javorja izkazala *T. versicolor*. LM in meritve debelin celičnih sten so v omenjenih primerih nakazale značilen razkroj celuloze in hemiceluloze vzorcev lesa in na tak način potrdile veliko občutljivost lesa gorskega javorja za omenjeno glivo, povzročiteljico bele trohnobe. Znatne poškodbe zaradi delovanja *T. versicolor* so bile v nadaljevanju opazne in potrjene tudi s pomočjo SEM.

Za oba izolata *E. parasitica*, vključena v poskus, smo s SEM ugotovili manjšo učinkovitost preraščanja in razkroja lesa. Prečni in tangencialni prerezi vzorcev lesa, ki so bili v poskusu izpostavljeni izolatoma *E. parasitica*, so kazali značilno manjši vpliv omenjenih izolatov na razkroj lesa. To se sklada tudi z rezultati o manjših izgubah mas in debelejših celičnih stenah v primerjavi z delovanjem *T. versicolor*. Izolata *E. parasitica* sta bila sposobna naselitve javorjevega lesa, vendar sta bila manj uspešna pri razkroju oz. sta povzročila razkroj manjših razsežnosti. Meritve debelin celičnih sten vlaken in trahej v vzorcih, izpostavljenih izolatoma *E. parasitica,* se znotraj posamezne branike niso značilno razlikovale. V našem primeru smo ugotovili značilne razlike v povprečni izgubi mase med vzorci, izpostavljenimi

različnima izolatoma *E. parasitica*, medtem ko French (1969) ni zaznal omenjenih razlik. Na podlagi rezultatov obarvanja prerezov lesa s safranin (lignin obarva rdeče)/astra modrim (celulozo obarva modro) barvilom, ki je notranje dele sekundarnih celičnih sten obarval nekoliko modro, predvidevamo, da je *E. parasitica* sposobna razkroja lignina in jo lahko zato uvrstimo med glive, povzročiteljice bele trohnobe. Enako so delovanje *E. parasitica* v poznejših fazah razkroja označili tudi Pildain in sod. (2005), medtem ko naj bi v začetnih fazah razkroja vrsta povzročala mehko trohnobo (Worrall in sod., 1997; Pildain in sod., 2005). Pri navajanju tovrstnih zaključkov moramo biti sicer precej previdni, saj vemo, da *E. parasitica* ni ravno zelo agresivna pri razkroju lesa. Za točnejšo določitev tipa trohnobe bi bile nujne še dodatne analize encimske aktivnosti v različnih razmerah okolja, kar pa je presegalo okvir naše raziskave. Na splošno so zaprtotrosnice večinoma kategorizirane kot glive, ki povzročajo mehke trohnobe (Mäkelä in sod., 2015) in so kot takšne sposobne razkroja polisaharidov v začetnih fazah razkroja in lignina nekoliko pozneje (Brischke in Unger, 2017).

Obilje hif na sredini vzorcev lesa, kjer smo odvzeli tudi prečne in tangencialne prereze za mikroskopijo, kaže na dobro preraščanje in uspešno prodiranje glivnega micelija v tkivu lesa. Velika količina hif kaže na velik uspeh naselitve in posledičen glivni razkroj lesa. V primeru enega izmed izolatov *E. parasitica*, kjer smo hife zaznali le bolj ali manj na robu vzorca, predvidevamo, da izolat ni preveč učinkovito napredoval v les zaradi manjše sposobnosti prodiranja in kratkega časa trajanja izpostavitve. Tudi French (1967) je poročal o prodiranju *E. parasitica* v vlakna in traheje, občasno celo v parenhim trakov. Ni pa opazil nekega očitnega obarvanja ali razbarvanja lesa, kar se sklada tudi z našimi ugotovitvami. Pri vzorcu, ki je bil izpostavljen izolatu *E. parasitica* ZLVG 34 (Zbirka živih kultur Laboratorija za varstvo gozdov na Gozdarskem inštitutu Slovenije), smo z LM opazili le manjše znake glivnega delovanja. SEM pa je odkril prisotnost glivnih hif v trahejah, kar potrjuje domnevo o večji občutljivosti slednjega tipa mikroskopije. Čeprav LM omogoča hiter pregled velikega števila celic in lažjo pripravo vzorcev, jih je za zanesljivo določitev prisotnosti gliv smiselno pregledati še s SEM (Wilcox, 1993). Še posebno primerno je oba tipa mikroskopije kombinirati, ko želimo preverjati razkroj v začetnih fazah ali kadar se ukvarjamo z manj agresivnimi glivami. Podatki o majhnih izgubah mase in pojavnosti hif *E. parasitica* v vzorcih lesa se skladajo z opaženim počasnim napredovanjem vrste in razkrojem lesa na terenu. Povprečna letna rast glive v dolžino namreč znaša 1–2 cm (Sinclair in sod., 1987). Napredovanje glive je hitrejše v vzdolžni kot pa prečni smeri (Ogris in sod., 2009). Okuženi starejši javorji lahko z glivo živijo in rastejo več desetletij (Ogris in sod., 2005). Zaradi napredujoče glivne aktivnosti, razkroja lesa in izgube mehanskih lastnosti se starejša okužena drevesa pogosto prelomijo na mestu rakov (French, 1967; Kliejunas in Kuntz, 1974).

Izmerjene debeline celičnih sten so bile dobro povezane z izgubami mase vzorcev lesa. Uspešna naselitev in učinkovit razkroj lesnih tkiv sta se kazala v večjih izgubah lesne mase.

Najočitneje je bilo to pri vzorcih, izpostavljenih glivi *T. versicolor*. Čeprav so izolati *E. parasitica* povzročili približno štirikrat manjše izgube mase lesa kot izolata *T. versicolor*, ne gre zanemariti njenega vpliva na razkroj javorjevega lesa. Ker *E. parasitica* navadno okuži najnižji del debla, ki je hkrati tudi najdebelejši in najvrednejši del drevesa, je vsaka še tako majhna škoda nezaželena. Že začetni razkroj lesa lahko namreč pomeni značilno zmanjšanje mehanskih lastnosti (Schmidt, 2006).

Gostota lesa lahko glede na literaturo določa naravno odpornost lesa na delovanje gliv. Obstajajo raziskave, ki potrjujejo, da se manjše gostote lesa kažejo v večji občutljivosti za razkroj lesa z glivami (Humar in sod., 2008; Dadzie in Amoah, 2015). Podobno bi lahko sklepali tudi za rezultate v naši študiji. Predvidevamo, da je javorjev rani les bolj občutljiv za naselitev z glivami in za razkroj. Rani les je v primerjavi s prehodnim in poznim lesom kazal nekoliko tanjše celične stene trakov in trahej, kar se ujema z nekoliko manjšimi gostotami (Shigo in Marx, 1977). Na podlagi izračunanih vrednosti gostot lesa in vsebnosti vode sklepamo, da ima maklenov les nekoliko drugačne lastnosti kot preostali vrsti javorjev, ki smo ju uporabili v raziskavi (gorski in ostrolistni javor). Gostota lesa in vsebnost vode pri maklenu sta bili namreč značilno večji. Vendar pa povezava med vsebnostjo vode in gostoto lesa ni bila značilna. Večja gostota maklenovega lesa se v večini primerov ni odražala v manjši občutljivosti za razkroj in posledično manjših izgubah lesa. To kaže na kompleksnost procesa razkroja lesa, kjer pomembno vlogo igra tudi odnos med glivo in gostiteljskim drevesom.

Obarvanje in razkroj lesa sta večinoma posledica delovanja bakterij ali gliv, ki vstopajo skozi odmrle veje v živo tkivo (Shigo, 1975). Značilnosti in potek razkroja lesa dobro opisuje model CODIT, ki sta ga razvila Shigo in Marx (1977) in smo ga predstavili v uvodnem poglavju. Green in sod. (1981) so na rdečem javorju proučevali kompartmentalizacijo obarvanja in razkroja lesa, ki je posledica vstopa mikroorganizmov skozi odmrle veje. Ločili so dva osnovna tipa odmrlih vej: 1) veje z jasno izraženo barierno cono zelene barve, ki ločuje obarvan in razkrojen les od beljave v deblu in 2) veje, ki nimajo barierne cone in se zato obarvan les širi v deblo. Ugotovili so, da odsotnost ali uničenje omenjene cone olajša širjenje mikroorganizmov, obarvanja in razkroja lesa v deblo. Oblikovanje barierne cone je izjemno pomembno tudi v primeru okužb z *E. parasitica*. *Eutypella parasitica* namreč po doslej znanih podatkih vstopa v gostitelja skozi odmrle veje ali poškodbe na deblu in tako sproži bolj ali manj uspešno kompartmentalizacijo. Če se barierna cona ne oblikuje, lahko gliva nemoteno napreduje v les debla in povzroča značilno obarvanje in razkroj lesa.

3.2 SKLEPI

Z raziskavo najpogostejših gliv v lesu odmrlih vej gorskega javorja smo določili izjemno širok spekter gliv. Večinoma so bili saprofiti iz debla Ascomycota. Pestrost ugotovljenih taksonov je še toliko pomembnejša, če upoštevamo dejstvo, da so bile lokacije vzorčenj

relativno blizu. Pri interpretaciji številčnosti izoliranih in prepoznanih gliv pa velja opozoriti, da je v vzorcih zagotovo še več vrst, ki jih zaradi počasne rasti ali neustreznih pogojev izolacije in inkubacije nismo zaznali. Za taksone, ki smo jih označili kot "sp." in določili le do nivoja rodu ali celo višje, bi bile potrebne dodatne morfološke in molekularne analize, ki bi razvozlale njihovo identiteto.

Poleg številnih prepoznanih vrst smo zaznali tudi veliko pestrost vrst in majhno podobnost glivnih združb. Pestrost vrst smo določili kot funkcijo števila različnih vrst in številčnosti posamezne vrste (Magurran, 2004). Pri proučevanju glivnih združb pa smo se osredotočili le na prisotnost oz. odsotnost določene vrste (Jaccard, 1908). Razlike v pestrosti in podobnosti glivnih združb smo proučevali za različne lokacije, različna mesta izolacij in različne debelinske razrede vej. S pomočjo Kruskall-Wallisovega testa na podlagi vrednosti Shannonovega diverzitetnega indeksa nismo ugotovili razlik v pestrosti vrst med različnimi lokacijami vzorčenja, med različnimi mesti izolacij in med različnimi debelinskimi razredi vej. Na podlagi Jaccardovega indeksa podobnosti lahko potrdimo prvo hipotezo (H1) o značilnih razlikah v glivni združbi med različnimi lokacijami vzorčenja, med različnima mestoma izolacije in med različnimi debelinskimi razredi vej. Prav tako lahko potrdimo tudi drugo hipotezo (H2) o vrednostih Shannonovega diverzitetnega indeksa nad 3 med različnimi lokacijami vzorčenja, mestoma izolacije in različnimi debelinskimi razredi vej.

Najpogosteje izolirane vrste gliv v lesu odmrlih vej gorskega javorja so bile *Eu. maura*, *Eutypa* sp., *F. avenaceum*, *N. acerina* in *E. parasitica*. Med najpogosteje izoliranimi vrstami je samo *Eu. maura* tako univerzalna, da se pojavlja tudi v drugih, tujih študijah vrst na vejah gorskega javorja.

Med najpogosteje izoliranimi vrstami je bila tudi *E. parasitica*, povzročiteljica javorovega raka. Glede na tak rezultat sklepamo na večjo razširjenost glive, kot je bilo znano doslej. Eden izmed zanimivejših rezultatov je bila izolacija *E. parasitica* iz vseh vzorčnih lokacij, čeprav smo simptome okužb in razvite rake opazili le na treh lokacijah od petih. To dejstvo odpira številna dodatna vprašanja in sili k razmišljanju o razlogih. Na podlagi naših rezultatov in rezultatov iz prejšnjih raziskav predvidevamo, da so bile okužbe izredno mlade in se zato na javorjih še niso razvili značilni znaki raka. Lahko bi govorili celo o asimptomatskih okužbah, vendar pa moramo biti pri tem izjemno previdni, saj ne gre zamenjevati šibkega oz. počasnega patogena z endofitom. Z raziskavo nismo odkrili razlik v pestrosti vrst ali združbi gliv med vejami gorskega javorja, kjer se je pojavljala *E. parasitica* in kjer je nismo izolirali. Ugotavljamo, da torej nobena izmed najpogosteje izoliranih vrst ni strogo povezana s pojavom ali odsotnostjo *E. parasitica*. Dodatno smo ugotovili še pogostejše izolacije *E. parasitica* iz razbarvanega dela lesa v deblu kot pa v zunanjem delu odmrle veje. Ta podatek smo povezali z veliko konkurenco, ki vlada v zunanjem delu veje. Predvidevamo, da se *E. parasitica* morebitnemu neuspešnemu tekmovanju izogne s hitrim napredovanjem v deblo.

Naša hipoteza (H3) o šibkejši konkurenčnosti *E. parasitica* v primerjavi z drugimi glivami v lesu odmrlih vej gorskih javorjev se je izkazala za delno pravilno. *Eutypella parasitica* ni v nobenem primeru prerasla micelija izzivalnega izolata. Ugotovili smo, da je v vseh dvojnih kulturah sicer nastal stik micelijev. Najpogosteje se je rast *E. parasitica* po stiku z drugim micelijem ustavila ali pa je izzivalni izolat delno prerasel micelij glive *E. parasitica* po začetnem stiku micelijev in ustavitvi rasti. Redkeje pa se je rast ustavila na razdalji brez stika micelija, kar bi bila lahko posledica sproščanja sekundarnih metabolitov z zaviralnim učinkom.

Izzivalni izolati so glede na vrednosti antagonističnega indeksa (AI) šibki antagonisti. Najvišje vrednosti smo beležili pri *Eutypa* sp., *Eu. maura*, *Neonectria* sp. in *P. incarnata*. Omenjeni izolati so značilno negativno vplivali na rast *E. parasitica* v dvojni kulturi in jih lahko označimo za najbolj tekmovalno uspešne izolate v naši raziskavi. Reizolacije *E. parasitica* iz dvojnih kultur z *Eutypa* sp., *F. avenaceum* in *Neonectria* sp. so bile neuspešne. Omenjeni izolati so najverjetneje v interakcijski coni premagali in nadomestili *E. parasitica*. Na drugi strani pa so bile reizolacije *Diaporthe* sp., *N. acerina* in *Pe. irregularis* manj uspešne. V takih primerih sklepamo na večji tekmovalni uspeh *E. parasitica*. Na podlagi vrednosti AI in uspešnosti reizolacij (s) lahko štejemo *E. parasitica* za šibkejšo tekmovalno vrsto, ki ne vloži veliko energije v neposredno tekmovanje z drugimi vrstami.

S testom dvojnih kultur smo pridobili delni vpogled v kompleksnost interakcij med *E. parasitica* in najpogosteje izoliranimi vrstami gliv v lesu odmrlih vej gorskega javorja. *Eutypa* sp., *Eu. maura*, *Neonectria* sp. in *P. incarnata* so se izkazale za najbolj obetavne vrste za uporabo v sklopu biološke kontrole *E. parasitica*.

V prvem delu raziskave smo določili najpogostejše vrste gliv v odmrlih vejah gorskega javorja, ki je glede na dosedanje raziskave najpogostejše vstopno mesto za vstop *E. parasitica* v gostitelja. V nadaljevanju smo proučevali medsebojni vpliv in morebitni antagonizem najpogostejših vrst gliv v lesu odmrlih vej na glivo *E. parasitica*. Pridobili smo razširjeno znanje o izoliranih vrstah in njihovem vplivu na uspešnost naselitve in rast glive *E. parasitica* v dvojnih kulturah. Vendar pa moramo biti pri interpretaciji in posploševanju naših rezultatov izjemno previdni; gre namreč za preliminarne rezultate laboratorijskih testov, ki bi jih bilo nujno razširiti z več ponovitvami posameznih kombinacij, z več uporabljenimi izolati *E. parasitica*, z dodatnimi raziskavami sekundarnih metabolitov in njihovega morebitnega antagonističnega vpliva (Yuen in sod., 1999; Prior in sod., 2017). Za točnejše zaključke o uporabi določenih vrst za biološko kontrolo *E. parasitica* pa bi bilo nujno raziskati tudi značilnosti interakcij v naravnem okolju. Zagotovo pa so ugotovljeni rezultati dobra osnova za nadaljnje študije.

Našo četrto hipotezo (H4) o počasnem razkroju lesa javorjev zaradi delovanja *E. parasitica* v primerjavi z drugimi trohnobnimi glivami, ki smo jih vključili v poskus, lahko potrdimo.

Gliva *E. parasitica* je povzročila manjše izgube lesne mase gorskega javorja (*A. pseudoplatanus*), ostrolistnega javorja (*A. platanoides*) in maklena (*A. campestre*) v primerjavi z obema referenčnima glivama razkrojevalkama, tj. *Trametes versicolor* in *Gloeophyllum trabeum*. Vrste, vključene v poskus, so kazale različne potenciale razkroja lesa. Kot najučinkovitejši sta se izkazali referenčni vrsti *T. versicolor* in *G. trabeum*, znani povzročiteljici bele oz. rjave trohnobe. Velike izgube mase lesa najpogosteje rastočih javorjev v Sloveniji kažejo na njihovo občutljivost za delovanje omenjenih vrst gliv. Preostale vrste (*Diaporthe* sp., *Eutypa* sp., *Eu. maura*, *E. parasitica*, *Fusarium* sp., *N. acerina*, *Ne. faginata* in *N. punicea*) so bile manj učinkovite. Pri vizualnem pregledu vzorcev ni bilo opaziti znakov obarvanja lesa.

S statistično obdelavo podatkov smo odkrili nekaj značilnih povezav med izračunanimi parametri. Večje izgube mase smo povezali z večjo vsebnostjo vode v vzorcih. Prav tako so se večje izgube mase kazale v tanjših celičnih stenah vlaken in trahej. Zaznali smo tudi povezavo med izgubami mase in gostotami lesa. Manjše gostote so pomenile večjo občutljivost za razkroj lesa in s tem večje izgube mase.

Poleg izračunov izgub mase, vsebnosti vode in gostot smo s pomočjo svetlobne in vrstične elektronske mikroskopije opazovali tudi strukturne spremembe v vzorcih, izpostavljenih delovanju izbranih vrst gliv. Z mikroskopijo smo potrdili rast glivnih hif v trahejah in v določenih primerih tudi očiten razkroj celičnih sten.

Počasen razkroj lesa zaradi delovanja *E. parasitica* lahko povežemo tudi s počasnim napredovanjem glive v lesu. Čeprav je *E. parasitica* povzročila značilno manjše izgube mase kot najuspešnejši vrsti (*T. versicolor* in *G. trabeum*), vključeni v poskus, ne smemo zanemariti njenega vpliva na razkroj javorjevega lesa, še posebno na daljši rok. Ne smemo pozabiti tudi na povsem drugačne razmere, ki vladajo v naravi in jih v laboratorijskih pogojih nismo mogli poustvariti. Vsekakor pa je ta del raziskave zagotovil splošen pregled aktivnosti razkroja *E. parasitica* v lesu naših najpogostejših vrst javorjev. Na podlagi rezultatov obarvanja vzorcev sklepamo, da *E. parasitica* razkraja lignin in jo lahko kot tako uvrščamo med glive povzročiteljice bele trohnobe.

Na podlagi pridobljenih rezultatov lahko oblikujemo naslednje zaključke:

- V celoti potrdimo prvo hipotezo (H1) – Glivne združbe v lesu odmrlih vej gorskega javorja (*A. pseudoplatanus*) se značilno razlikujejo med različnimi lokacijami vzorčenja v osrednji Sloveniji (Rožnik, Smrekovec, Mokrc, Mala voda, Samotorica), med različnimi mesti izolacije (veja, deblo, kontrola) in med različnimi debelinskimi razredi vej (od 0,2 do 0,7 cm, od 0,8 do 1,2 cm, od 1,3 do 3,2 cm).
- V celoti potrdimo drugo hipotezo (H2) – Vrednost Shannonovega diverzitetnega indeksa vrst gliv izoliranih iz lesa odmrlih vej gorskega javorja (*A. pseudoplatanus*) med različnimi lokacijami vzorčenja v osrednji Sloveniji (Rožnik, Smrekovec,

Mokrc, Mala voda, Samotorica), med različnima mestoma izolacije (veja, deblo) in med različnimi debelinskimi razredi vej (od 0,2 do 0,7 cm, od 0,8 do 1,2 cm, od 1,3 do 3,2 cm) je višja od 3.

- Deloma ovržemo tretjo hipotezo (H3) – Gliva *E. parasitica* je v čisti kulturi šibko konkurenčna desetim najpogosteje izoliranim vrstam gliv v lesu odmrlih vej gorskega javorja (*A. pseudoplatanus*). Hipotezo ovržemo v dvojnih kulturah z *Diaporthe* sp., *N. acerina* in *Pe. irregularis*, potrdimo pa jo lahko v preostalih testiranih dvojnih kulturah z *Eutypa* sp., *Eu. maura*, *E. parasitica*, *F. avenaceum*, *Neonectria* sp., *P. incarnata* in *Ph. pustulata*.
- V celoti potrdimo četrto hipotezo (H4) – Gliva *E. parasitica* povzroči manjše izgube lesne mase gorskega javorja (*A. pseudoplatanus*), ostrolistnega javorja (*A. platanoides*) in maklena (*A. campestre*) v primerjavi z glivama razkrojevalkama *Trametes versicolor* in *Gloeophyllum trabeum*.

Eutypella parasitica je relativno slabo raziskana vrsta. Z našimi poskusi smo podrobneje raziskali izbrana področja njene biologije in na tak način prispevali k razširitvi znanja o njenem delovanju. Ugotovili smo zanimive in uporabne zaključke, vendar pa je še veliko prostora za nadaljevanje raziskav na tem področju. V nadaljevanju izpostavljamo nekaj najpomembnejših predlogov:

- določitev manj pogostih vrst v odmrlih vejah gorskega javorja;
- več vzorčnih lokacij, ki bi omogočale zanesljivejše primerjave združb, predvsem med lokacijami z *E. parasitica* in brez nje;
- uporaba več izolatov *E. parasitica* in več ponovitev posameznih kombinacij v dvojnih kulturah za preskus antagonizma oz. tekmovalnosti;
- uporaba dodatnih izzivalnih vrst, ki so v literaturi navedene kot antagonisti rodu *Eutypella* spp. v dvojnih kulturah;
- raziskava sekundarnih metabolitov in njihovega vpliva na antagonistično dejavnost;
- raziskava interakcij *in vivo*;
- raziskava vpliva gliv iz rodu *Neonectria* spp. na *E. parasitica*;
- raziskava napredovanja *E. parasitica* v lesu in tvorba bariernih con;
- natančnejša določitev tipa trohnobe, ki ga povzroča *E. parasitica*;
- dodatne raziskave glede obarvanja in razkroja lesa v naravi;
- raziskati, ali je *E. parasitica* endofit.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Javorov rak, ki ga povzroča gliva *Eutypella parasitica*, so odkrili in opisali leta 1937 na meji med Kanado in Združenimi državami Amerike (Davidson in Lorenz, 1938). V Evropi je bil javorov rak prvič zabeležen v Sloveniji leta 2005 (Jurc in sod., 2006). *Eutypella parasitica* je po definiciji invazivna tujerodna vrsta, ki se je pri nas ustalila, povzroča gospodarsko škodo in se uspešno razmnožuje. Gliva najpogosteje okuži gostitelja skozi odmrlo vejo ali poškodbo na deblu (French, 1967). Do naših raziskav je veljalo, da ima vrsta izjemno velik infekcijski potencial, vendar so opazne okužbe relativno maloštevilne.

Kot glavni namen pričujoče disertacije smo si zadali podrobneje raziskati izbrana področja biologije vrste *E. parasitica*, ki povzroča javorov rak, njenega vpliva na izgubo mase lesa treh vrst javorjev in odnosov z drugimi najpogostejšimi vrstami gliv, ki se pojavljajo v odmrlih vejah gorskega javorja. Opredelili smo tri raziskovalne cilje.

Cilj 1 (C1): Določiti najpogostejše vrste gliv v odmrlih vejah gorskega javorja na petih lokacijah v okolici Ljubljane.

Cilj 2 (C2): Izmeriti vpliv desetih najpogostejših vrst gliv v odmrlih vejah gorskega javorja na rast glive *E. parasitica* v čisti kulturi in ugotoviti njihov morebitni antagonizem.

Cilj 3 (C3): Ugotoviti izgubo lesne mase treh vrst javorjev (*Acer pseudoplatanus*, *A. platanoides*, *A. campestre*) zaradi glive *E. parasitica* po metodi mini-blok, ki temelji na standardu SIST EN 113:2002, in primerjati rezultate z izolati gliv *Trametes versicolor*, *Gloeophyllum trabeum* in s petimi najpogostejšimi glivami, ki povzročajo trohnobo v javorjevih odmrlih vejah.

Na petih vzorčnih lokacijah v okolici Ljubljane smo od novembra 2017 do marca 2018 vzorčili odmrle veje gorskega javorja. Na vsaki lokaciji smo odvzeli po 40 vzorcev (skupaj 200 vej), ki smo jih v laboratoriju površinsko sterilizirali in koščke z različnih mest odmrle veje in debla ($n_{MAX} = 20$) nacepili na 2 % gojišče MEA (gojišče z ekstraktom ječmenovega slada; angl. malt extract agar). S periodičnim pregledovanjem in precepljanjem smo glive izolirali v čiste kulture. Na podlagi makromorfoloških značilnosti smo kulture razvrstili v morfotipe. Najpogostejše morfotipe (število kultur > 5) smo določili do nivoja vrste ali rodu z analizo nukleotidnih zaporedij izbranih molekularnih markerjev (del odsekov ITS rDNA, EF-1α). Na podlagi podatkov smo izračunali relativne frekvence prepoznanih taksonov, indeks gostote, Shannonov diverzitetni indeks, Jaccardov indeks podobnosti ipd. V nadaljevanju smo preverjali razlike v pestrosti vrst in podobnosti glivnih združb med različnimi lokacijami vzorčenja, različnimi mesti izolacij in različnimi debelinskimi razredi

vej. Kulture smo deponirali v ZLVG, pridobljena nukleotidna zaporedja pa v podatkovno zbirko GenBank.

V nadaljevanju smo glivo *E. parasitica* izpostavili desetim najpogosteje izoliranim vrstam gliv v lesu odmrlih vej gorskega javorja v dvojnih kulturah na 3,9 % gojišču PDA (krompirjev agar z glukozo; angl. potato dextrose agar). Pripravili smo tudi samoinhibicijske (isti izolat *E. parasitica*) in kontrolne (*E. parasitica* in sterilen agar) dvojne kulture. Vsako kombinacijo smo ponovili trikrat. Rast smo spremljali v petrijevkah, določili tip interakcije in izračunali antagonistični indeks (AI). Po desetih dneh od stika micelijev oz. deset dni po koncu rasti na navidezni vodoravni liniji med nacepljenima koščkoma smo izvedli reizolacije (pet koščkov iz interakcijske cone in dva kontrolna koščka). Razvite kulture smo morfološko pregledali in z molekularnimi metodami potrdili prisotnost oz. odsotnost *E. parasitica* v reizoliranih kulturah ter izračunali uspešnost reizolacij (s) iz interakcijske cone.

Na podlagi prilagojenega standarda EN 113 smo vzorce lesa (30 × 10 × 5 mm) *A. pseudoplatanus*, *A. platanoides* in *A. campestre* za primerjavo izpostavili štirim izolatom *E. parasitica* in devetim drugim glivnim vrstam. Vzorce lesa smo stehtali v absolutno suhem stanju pred izpostavitvijo (m_0), v vlažnem končnem stanju (m_1) in v absolutno suhem stanju po izpostavitvi (m_2). Na podlagi meritev smo izračunali povprečne izgube mas in vsebnost vode v vzorcih. Strukturne spremembe v lesu smo si ogledali s pomočjo svetlobne (LM) in vrstične elektronske mikroskopije (SEM). Na prečnih prerezih izpostavljenih vzorcev lesa smo s pomočjo LM izmerili in izračunali povprečne debeline celičnih sten vlaken in trahej.

Izmed 91 najpogosteje izoliranih morfotipov v lesu odmrlih vej gorskih javorjev smo določili 58 različnih taksonov gliv. Najpogosteje izolirane vrste so bile *Eutypa maura*, *Eutypa* sp. 2, *Fusarium avenaceum*, *Neocucurbitaria acerina* in *E. parasitica*. Razlike v pestrosti vrst med različnimi lokacijami vzorčenja, med različnimi mesti izolacije in med različnimi debelinskimi razredi vej so bile neznačilne. Ravno obratno pa smo ugotovili značilne razlike v glivnih združbah med različnimi lokacijami vzorčenja, med različnimi mesti izolacij in med različnimi debelinskimi razredi vej. *Eutypella parasitica* smo izolirali tudi na lokacijah, kjer predhodna inventura javorjev ni pokazala prisotnosti javorovega raka. Ugotovili smo pogostejše izolacije *E. parasitica* iz obarvanega lesa v deblu kot iz zunanjega dela odmrle veje, kar lahko kaže na njeno manjšo konkurenčnost in umik pred drugimi, bolj tekmovalnimi vrstami. Primerjava pestrosti glivnih vrst med odmrlimi vejami z *E. parasitica* in brez nje pa ni pokazala značilnih razlik. Zato domnevamo, da nobena izmed vrst ni strogo povezana s pojavljanjem *E. parasitica*.

V dvojnih kulturah je v skoraj vseh primerih nastal stik micelijev med *E. parasitica* in izzivalnim izolatom. Najpogosteje se je rast *E. parasitica* po stiku z drugim micelijem ustavila ali pa jo je izzivalni izolat delno prerasel. Največje vrednosti AI in s tem značilno negativen vpliv na rast *E. parasitica* v dvojnih kulturah smo zabeležili pri *Eutypa* sp., *Eu.*

maura, *Neonectria* sp. in *Peniophora incarnata*. Hkrati so imele omenjene vrste veliko uspešnost reizolacij iz interakcijske cone in jih lahko označimo kot potencialno uporabne v sklopu biološke kontrole *E. parasitica*. Na drugi strani so bile reizolacije *Diaporthe* sp., *N. acerina* in *Petrakia irregularis* manj uspešne, domnevno zaradi večjega tekmovalnega uspeha *E. parasitica*. S testom dvojnih kultur in preverjanjem uspešnosti reizolacij smo pridobili delni vpogled v kompleksnost interakcij med *E. parasitica* in najpogosteje izoliranimi vrstami gliv v lesu odmrlih vej gorskega javorja. Pri interpretaciji in posploševanju rezultatov moramo biti izjemno previdni. Preliminarne rezultate laboratorijskih testov bi bilo treba razširiti in tako pridobiti verodostojnejše informacije o medsebojnemu učinkovanju vrst, morebitnem antagonizmu in prenosu ugotovitev v prakso.

Po 15-tedenski inkubaciji vzorcev lesa v petrijevkah z glivami sta izguba mase lesa in mikroskopska analiza pokazali dobro razraščanje gliv in različne potenciale razkroja lesa. Pri razkroju lesa in posledični izgubi mase sta bili najuspešnejši referenčni vrsti gliv *Trametes versicolor* in *Gloeophyllum trabeum*, ki sta znani razkrojevalki in povzročiteljici bele oz. rjave trohnobe po vsem svetu. V povprečju so glive povzročile najmanjšo izgubo mase pri vzorcih *A. pseudoplatanus* in največjo pri vzorcih *A. campestre*, kar lahko delno povežemo z razlikami v gostoti lesa. *Eutypella parasitica* je povzročila največje izgube lesne mase pri vzorcih gorskega javorja (povprečje 6,6 %). Povprečna izguba lesne mase treh vrst javorjev zaradi delovanja izolatov *E. parasitica* je znašala 5,5 %, iz česar sklepamo, da je gliva počasna razgrajevalka lesa. Pri vseh izolatih smo odkrili statistično značilno pozitivno korelacijo med izgubo mase in vsebnostjo vode ter značilno negativno korelacijo med izmerjeno debelino celičnih sten in izgubo mase vzorcev. Statistična analiza je pokazala značilne razlike v izgubi mase in vsebnosti vode med različnimi izolati *E. parasitica*. Na podlagi obarvanja tkiv s safranin/astra modrim barvilom domnevamo, da je *E. parasitica* sposobna razkroja lignina in bi jo zato lahko uvrstili med povzročiteljice bele trohnobe lesa. Za dokončno potrditev te trditve bi bile nujne še dodatne raziskave.

Glede na postavljene cilje raziskave smo ugotovili zaključke, ki jih navajamo v nadaljevanju. Uspešno smo dosegli vse tri postavljene cilje. V sklopu prvega (C1) smo uspešno določili najpogosteje izolirane vrste gliv, ki se pojavljajo v lesu odmrlih vej gorskega javorja na petih lokacijah v okolici Ljubljane. V nadaljevanju smo ugotavljali medsebojni vpliv in morebitni antagonizem najpogostejših vrst gliv v lesu odmrlih vej gorskega javorja na glivo *E. parasitica*. Potrdili smo našo domnevo o šibkejši konkurenčnosti *E. parasitica* v dvojnih kulturah z *Eutypa* sp., *Eu. maura*, *Neonectria* sp. in *P. incarnata*, medtem ko je imela v dvojnih kulturah z *Diaporthe* sp., *N. acerina* in *Pe. irregularis* večji tekmovalni uspeh. Cilj (C2) je bil torej uspešno dosežen, domneva pa delno potrjena. V tretjem delu poskusa smo preverjali vpliv *E. parasitica* na razkroj lesa naših treh najpogostejših vrst javorjev. Ugotovili smo, da *E. parasitica* povzroča manjšo izgubo mase in s tem razkroj lesa javorjev kot uporabljeni referenčni vrsti (*T. versicolor* in *G. trabeum*). Tako smo uspešno dosegli še

tretji raziskovalni cilj (C3) in potrdili našo domnevo o počasnejšem razkroju lesa zaradi delovanja *E. parasitica* v primerjavi z drugimi trohnobnimi glivami.

Z našo raziskavo smo pridobili razširjeno znanje o vrstah, ki se pojavljajo v odmrlih vejah gorskega javorja, ki je hkrati tudi najpogostejše vstopno mesto za *E. parasitica*. Zaradi vpliva drugih vrst na uspešnost naselitve in razrasti glive *E. parasitica* v odmirajočih javorjevih vejah smo predlagali potencialno uporabne vrste za biološko kontrolo javorovega raka. Ugotavljali smo tudi vpliv *E. parasitica* na razkroj lesa, ki je sicer počasna, vendar je ne smemo zanemariti. Gliva namreč v večini primerov poškoduje spodnji in tako tudi najdebelejši, najvrednejši sortiment, zato naši rezultati pripomorejo k boljšemu razumevanju vpliva glive na ekonomsko in tehnično razvrednotenje okuženih javorjev.

V doktorski disertaciji smo podrobneje obravnavali določena področja delovanja glive *E. parasitica*, ki povzroča javorov rak. Doslej je bilo relativno malo raziskav, ki bi obravnavale omenjeno vrsto. Z našimi rezultati smo prispevali k razširitvi vedenja o njeni biologiji. Zavedamo se, da so rezultati in zaključki le del, na določenih mestih še vedno slabo raziskanega mozaika. V prihodnje so torej še priložnosti za raziskovanje in razvijanje novih ugotovitev in znanj s tega področja.

4.2 SUMMARY

Eutypella canker of maple, which is caused by *Eutypella parasitica*, was discovered and described in 1937 on the border between Canada and the United States (Davidson and Lorenz, 1938). In Europe, Eutypella canker of maple was first recorded in Slovenia in 2005 (Jurc et al., 2006). *Eutypella parasitica* is an invasive alien species that has become established in our country, causes economic damage, and reproduces successfully. The fungus most frequently infects the host through a branch stub or bark wound (French, 1967). The species has an extremely high infectious potential, but visible infections are still relatively rare.

The main purpose of this dissertation was to investigate in detail the selected areas of biology of *E. parasitica*, the causative agent of Eutypella canker of maple, its impact on the mass loss of three maple species, and its relationships with other frequent fungal species that occur in the wood of the dead branches of sycamore maple. We defined three research objectives:

Objective 1 (O1): To determine the most frequent species of fungi in the dead branches of sycamore maple at five locations in the vicinity of Ljubljana.

Objective 2 (O2): To measure the influence of the ten most frequent species of fungi isolated from the dead branches of sycamore maple on the growth of the fungus *E. parasitica* in pure culture and to determine their possible antagonism.

Objective 3 (O3): To measure the mass loss of three species of maple (*Acer pseudoplatanus*, *A. platanoides*, *A. campestre*) due to the fungus *E. parasitica* using the miniblock method based on the SIST EN 113: 2002 standard and to compare the results with isolates of *Trametes versicolor*, *Gloeophyllum trabeum*, and the five most frequent fungi causing rot in dead maple branches.

Between November 2017 and March 2018, we sampled the dead branches of sycamore maple at five sampling sites in the vicinity of Ljubljana. Forty samples (200 branches in total) were collected at each site. Samples were surface sterilized in the laboratory, and subsamples from different isolation sources in each sample ($n_{MAX} = 20$) were plated on 2 % MEA (malt extract agar). The fungi were isolated into pure cultures by periodic inspection and sub culturing. Based on macromorphological characteristics, cultures were grouped into morphotypes. The most frequent morphotypes (number of cultures > 5) were determined up to the species or genus level by nucleotide sequence analysis of selected molecular markers (part of ITS rDNA sections, EF-1α). Based on the data, we calculated the relative frequencies of identified taxa, density index, Shannon diversity index, and Jaccard similarity index. Furthermore, we examined the differences in species diversity and fungal communities between different sampling sites, different isolation sources, and different branch thickness classes. Cultures were deposited in the culture collection of the Laboratory of Forest Protection at the Slovenian Forestry Institute (ZLVG), and the obtained sequences in the GenBank database.

The fungus *E. parasitica* was then exposed to the ten most frequently isolated species of fungi in the wood of the dead branches of sycamore maple in dual cultures on 3.9 % PDA (potato dextrose agar). Self-inhibitory (same isolate of *E. parasitica*) and control (*E. parasitica* and sterile agar) dual cultures were also prepared. Each combination was repeated three times. We monitored growth in Petri dishes, determined the type of interaction, and calculated the antagonism index (AI). Ten days after mycelium contact or ten days after the end of growth on the apparent horizontal line between the inoculated plugs, re-isolations were performed (five plugs from the interaction zone and two control plugs). Re-isolated cultures were morphologically examined, the presence or absence of *E. parasitica* was confirmed with molecular methods, and the success of re-isolations (s) from the interaction zone was calculated.

Based on the modified standard EN 113, wood samples (30 × 10 × 5 mm) of *A. pseudoplatanus*, *A. platanoides*, and *A. campestre* were exposed to four isolates of *E. parasitica* and nine other fungal species for comparison. Wood samples were weighed to obtain initial dry mass (m_0) before exposure, the mass of a wet sample (m_1), and final dry mass (m_2) after exposure. Based on the measurements, we calculated the average mass loss and moisture content of the samples. Structural changes in the wood were observed using light (LM) and scanning electron microscopy (SEM). On the cross-sections of exposed wood

samples, the average cell wall thicknesses of fibers and vessels were measured and calculated using LM.

Of the 91 most frequently isolated morphotypes in the wood of the dead branches of sycamore maple, 58 different fungal taxa were identified. The most frequently isolated species were *Eutypa maura*, *Eutypa* sp. 2, *Fusarium avenaceum*, *Neocucurbitaria acerina*, and *E. parasitica*. Differences in species diversity between sampling sites, between isolation sources, and between branch thickness classes were not significant. Conversely, we found significant differences in fungal communities between sampling sites, between isolation sources, and between branch thickness classes. *Eutypella parasitica* was also isolated from sites where a previous maple inventory did not show the presence of Eutypella canker of maple. We found more frequent isolations of *E. parasitica* from the discoloured wood of trunks than from the outer parts of dead branches, which may indicate its weaker competitiveness and withdrawal from other, more competitive species. However, a comparison of the fungal species diversity between dead branches with and without *E. parasitica* showed no significant differences, leading to the assumption that none of the species is strictly associated with the occurrence of *E. parasitica*.

In almost all dual cultures, the challenge isolate made mycelium contact with *E. parasitica*. The growth of *E. parasitica* most frequently stopped after contact with another mycelium or was partially overgrown by the challenge isolate. The highest values of AI and thus a significantly negative effect on the growth of *E. parasitica* in dual cultures were recorded in *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *Peniophora incarnata*. At the same time, those species had a high success of re-isolations from the interaction zone and can be characterized as potentially useful in the biological control of *E. parasitica*. On the other hand, the isolates of *Diaporthe* sp., *N. acerina*, and *Petrakia irregularis* were less successful, presumably due to the greater competitive success of *E. parasitica*. The dual culture test and verification of the success of re-isolations, gave us a partial insight into the complexity of the interactions between *E. parasitica* and the most frequently isolated fungal species in the wood of the dead branches of sycamore maple. We must be extremely careful when interpreting and generalizing the results. Preliminary results of laboratory tests should be expanded to provide more credible information on species interactions, possible antagonism, and translation of our findings into practice.

After 15 weeks of incubation of wood samples in Petri dishes with fungi, mass loss and microscopic analysis showed good fungal growth and different wood degradation potentials. *Trametes versicolor* and *Gloeophyllum trabeum*, well known as white and brown rot fungi worldwide, were the most successful in the decomposition of wood and the consequent loss of mass. On average, the tested fungi caused the lowest mass loss in *A. pseudoplatanus* samples and the highest loss in *A. campestre* samples, which can be partly attributed to differences in wood density. *Eutypella parasitica* caused the greatest mass loss in sycamore

maple samples (average 6,6 %). The average mass loss of the three maple species due to *E. parasitica* isolates was 5.5 %, based on which we conclude that the fungus is a slow degrader of wood. In all isolates, we found a statistically significant positive correlation between mass loss and moisture content and a characteristic negative correlation between the measured cell wall thickness and the mass loss of the samples. Statistical analysis showed significant differences in mass loss and moisture content between different *E. parasitica* isolates. Based on the staining of tissues with safranin/astra blue water solution, we assumed that *E. parasitica* was able to degrade lignin and could be considered as a white rot fungus. Further research is needed to definitively confirm this claim.

According to the set goals of the research, we can draw the following conclusions:
We successfully achieved all three goals. As part of the first (O1), we successfully identified the most frequently isolated species of fungi in the wood of the dead branches of sycamore maple at five sites in the vicinity of Ljubljana. Further on, we determined the interaction and possible antagonism between the most frequent fungal species in the wood of dead branches of sycamore maple and the fungus *E. parasitica*. We confirmed our assumption of the poor competitiveness of *E. parasitica* in dual cultures with *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata*. However, in dual cultures with *Diaporthe* sp., *N. acerina*, and *Pe. irregularis*, *E. parasitica* showed greater competitive success. Objective (O2) was therefore successfully achieved, and the assumption was partially confirmed. In the third part of the experiment, we examined the influence of *E. parasitica* on wood degradation in our three most common maple species. *E. parasitica* was found to cause lower mass loss and thus less efficient maple wood degradation than the reference species (*T. versicolor* and *G. trabeum*). Thus, we successfully achieved the third research goal (O3) and confirmed our assumption of the slower degradation of wood due to *E. parasitica* compared to the other three rot fungi.

Through our research, we gained extensive knowledge on the species that occur in the dead branches of sycamore maple, which is also the most likely entry point for *E. parasitica*. Due to the influence of other species on the efficiency of colonization and growth of *E. parasitica* in dead maple branches, we proposed potentially useful species for the biological control of Eutypella canker of maple. We also addressed the effect of *E. parasitica* on wood degradation, which is slow but not negligible. In most cases, the fungus damages the lower and thus the thickest, most valuable part of the tree. Our results therefore shed light on the impact of the fungus on the economic and technical devaluation of infected maples.

In the doctoral dissertation, we discussed in more detail certain areas of activity of the fungus *E. parasitica*, the causative agent of Eutypella canker of maple. To date, relatively little research has been done to address this species. With our results, we have contributed to broadening the knowledge on its biology. Our results and conclusions are only part of a mosaic that is still poorly researched in certain areas and there remain opportunities to research and develop new findings and knowledge in this field.

5 VIRI

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