Stephanie Gantz, Susanne Steudler, Hubertus Delenk, André Wagenführ, Thomas Bley

Zone line formation on artificial media and in hardwoods by basidiomycetes for production of spalted wood

Erstveröffentlichung in / First published in:

Holzforschung. 2017, 71(10), S. 833 – 841 [Zugriff am: 03.02.2020]. De Gruyter. ISSN 1437-434X.

DOI: https://doi.org/10.1515/hf-2016-0222

Stephanie Gantz*, Susanne Steudler, Hubertus Delenk, André Wagenführ and Thomas Bley

Zone line formation on artificial media and in hardwoods by basidiomycetes for production of spalted wood

DOI 10.1515/hf-2016-0222
Received November 30, 2016; accepted April 25, 2017; previously published online June 2, 2017

Abstract: One of the visual modification of wood is the formation of dark zone lines (ZLs) via interaction of fungi. The result is called spalted wood, which has hitherto been produced mainly in small batches. The main goal of the present study is to further develop techniques for rapid formation of ZLs in hardwoods. Various white rot and brown rot fungi were tested to this purpose. Initially, interactions of 148 combinations of 17 basidiomycetes in malt extract agar were evaluated and their antagonistic interactions were characterised in order to identify fungal pairs capable of rapidly forming high-quality ZLs. Six types of interactions were observed, among others; antibiosis and inhibition in contact, which differ in terms of variables including mycelial overgrowth and zone line formation. Furthermore, 23 pairs of ZL forming fungi on malt extract agar were identified. Then the interactions of five selected pairs of fungi grown on the hardwood species *Acer pseudoplatanus* L., *Betula pendula* Roth. and *Populus nigra* L. were examined to assess their utility for controlled mycological wood modification, also in terms of a possible substrate dependency of their interactions. The results indicate that *Lentinus tigrinus* fungus is one of the best and quickest producer of ZLs in mycological wood modification.

Keywords: antagonism, artificial media, basidiomycetes, fungal interactions, hardwood, *Lentinus tigrinus*, mycological wood modification, spalted wood, zone lines

***Corresponding author: Stephanie Gantz,** Technische Universität Dresden, Chair of Wood Technology and Fibre Materials Technology, Dresden, Germany, Phone: +49 351 463-38322, Fax: +49 351 463-38288, e-mail: stephanie.gantz@tu-dresden.de
Susanne Steudler and Thomas Bley: Technische Universität Dresden, Chair of Bioprocess Engineering, Dresden, Germany
Hubertus Delenk and André Wagenführ: Technische Universität Dresden, Chair of Wood Technology and Fibre Materials Technology, Dresden, Germany

Introduction

Wood properties can be modified by numerous methods including microbial or enzymatic treatments (Wagenführ and Scholz 2012). For example, in the 1950s and 1960s the so-called "Myko-Holz" was produced by means of basidiomycetes, which is a wood with highly uniform porosity and good impregnation qualities (Wagenführ and Luthardt 2012). Within the framework of decorative wood art, such as intarsia and artificial staining, modification by fungi provides some particularly attractive optical features, which are induced by wood-staining fungi. The products are known as spalted woods (Robinson et al. 2016), which are aesthetically very pleasant due to their typical mosaic appearance, which is especially popular among artists since the Middle Ages (Michaelsen et al. 1992). Spalting results from bleaching, pigmentation and (the focus of this study) zone line (ZL) formation (Robinson et al. 2007a, 2011a, 2016). ZLs, also called demarcation lines, are thin, irregular, dark layers in wood, which are caused amongst others by melanised hyphae (Schwarze et al. 1999; Robinson et al. 2007a; Tudor et al. 2013).

Hartig (1878) was the first, who described ZLs in a scientific paper. Hubert (1924) confirmed that ZLs are the results of wood decay. Harder (1911) studied the interactions between two different fungal cultures among various basidiomycetes and ascomycetes on agar cultures and observed effects like overgrowing, inhibition and antibiosis in the dual cultures (Rypáček 1966). The pairing experiments were repeated with different fungi, e.g. by Rypáček (1966) and Bertrand et al. (2013). In the early 1930s, ZLs in wood, artificial media and cotton-wool were classified as inter- and intra-species antagonism and fungal response to changes in the environment (Campbell 1933, 1934; Robinson et al. 2016). ZLs were also described as three-dimensional (3D) pseudosclerotial bodies (Campbell 1934; Lopez-Real 1975; Lopez-Real and Swift 1977).

In the 1970s, the increased interest on spalted wood gave new impetus to the scientific research. Melanin in the cell wall of fungi came into focus, which was extracted and characterised, and the similarity between melanin and indole (as the basic element of many natural dyes)

was detected (Ellis and Griffiths 1974, 1975). Lopez-Real (1975) found that ZLs also contain pseudosclerotial plates (PSP) and noted three stages of ZL formation: (1) proliferation of hyphae, (2) hyphal swelling and aggregation and (3) pigmentation. These steps occur in bladder-like cells (pseudosclerotial plate). It was also found that the fungi exhibit a similar melanised pseudosclerotium in various growth media. Soon it was also detected that ZL results from inter- and/or intrafungal antagonism (Campbell 1933; Robinson et al. 2007a; Tudor et al. 2013), thus the first in-detail investigations were performed via pairing of different fungal cultures. Adam and Roth (1967) applied this experimental set up as a diagnostic tool for genetically distinct fungi (Adam and Roth 1967).

A pioneering work was done by Phillips (1987), who created ZL on both agar plates and woods by antagonistic reactions between six white rot (WR) fungi, including the promising pairing *Trametes versicolor* and *Bjerkandera adusta* (Robinson 2012). Since the beginning of the late 2000s, the leading research group in the field of spalted wood worked at Brigham Young University and later at Michigan Technological University (Robinson 2012).

In the last decade, this working group focused on detection of suitable fungi and fungal pairings (Robinson et al. 2007a), as well as on methods of inoculation and incubation for reproducible production of spalted wood at the laboratory and the commercial scale (Robinson et al. 2009b, 2012; Robinson 2013). Methods of analysis were developed and the machinability and intensity of pigmentation (Robinson et al. 2007, 2009a) were investigated. Moreover, pigmentation was stimulated by addition of copper sulfate at various moisture and pH levels, and the effects of substrate and culture age were observed (Robinson and Laks 2010; Robinson et al. 2011a,b; Tudor et al. 2012, 2013).

Robinson et al. (2007a) tested 23 different WR species in 21 pairings regarding their ability of ZL formation in malt agar plates and maple blocks at intervals up to 16 weeks (Robinson et al. 2007a). The best results were obtained by pairing *T. versicolor/B. adusta* and *Polyporus brumalis/T. versicolor*. Other well-known ZL-forming fungi include *Armillaria mellea* (Schmidt 2006; Butin 2011), *Polyporus squamosus* (Schwarze et al. 1999; Schmidt 2006; Tudor et al. 2012) and *Xylaria polymorpha* (intrafungal antagonism) (Robinson and Laks 2010; Tudor et al. 2012). The incubation methodology for spalted wood production was also optimised. The soil in standard soil jar decay test (AWPA E10-09 2009) was replaced with vermiculite, which is a natural and more uniform substrate, and which is more homogenous in terms of chemical composition and physical properties (Robinson et al. 2009b). Furthermore, the block placement of the wood cubes (mostly 14 mm, rarer 37 mm) was investigated and it was observed that wood above vermiculite may increase pigmentation (Robinson et al. 2012).

The influence of additives and environmental characteristics were tested. Good pigmentation was observed in various hardwoods such as maple, beech, birch, aspen and basswood with pH between 4.5 and 5, and moisture contents (MCs) ranging from 22% to 96%, depending on the fungi in focus (Robinson et al. 2007b, 2012; Robinson and Laks 2010; Tudor et al. 2012, 2013). On some substrates, reduction of pigment formation was observed with increasing age of the culture (Robinson and Laks 2010). More ZL was developed, when *X. polymorpha* was grown on sugar maple treated with 1 kg m^{-3} copper sulfate (Robinson et al. 2011a). The pairing *T. versicolor/Scytalidium cuboideum* (interfungal) or *X. polymorpha/X. polymorpha* (intrafungal) on sugar maple was advantageous for commercial applications in combination with lateral dowel pin inoculation and liquid media inoculation (Robinson 2013; Tudor et al. 2013). The pairing *T. versicolor/Scytalidium cuboideum* and *X. polymopha* (different isolates) was studied on the substrates; beech, sugar maple, Norway maple and aspen with sample sizes of 81–96 cm in length and 15–25 cm in diameter (Robinson et al. 2013; Tudor et al. 2013). The inoculation method influenced the amount of ZL and pigmentation, i.e. more ZL was produced via lateral dowel pin inoculation, and more stain was produced by liquid media inoculation (Robinson et al. 2013; Tudor et al. 2013). Hitherto, only a few fungi have been identified as suitable producer for spalted wood (Robinson et al. 2013; Tudor et al. 2013).

The main objective of the present study was to identify more combinations of fungi capable of forming dark ZLs in wood. The reproducibility and the ZL formation speed and the aesthetical appearance of the spalted wood are important criteria for a good fungi combination (Koch 2014). Both new and well-known ZL-forming basidiomycetes will be tested under consideration of these selection criteria. The antagonistic interactions of fungi should be screened on malt extract ager plates, following the suggestions of Robinson et al. (2007a,b) and Bertrand et al. (2013). The inhibition between fungi can occur via contact inhibition or without a direct hyphal contact (antibiosis) (Witzany 2010; Bertrand et al. 2013). Mechanical repression (overgrowth) of one fungus by a stronger strain may be involved in competition between fungi (Witzany 2010). In focus will be the spalting of *Acer pseudoplatanus* L., *Betula pendula* Roth. and *Populus nigra* L. with 40% MC according to European standards.

Materials and methods

Microorganisms and pre-culture: All investigated fungi (Table 1) were maintained on 1.5% malt extract agar plates (prepared on a ready-to-use agar mixture from Carl Roth GmbH + Co. KG, Karlsruhe, Germany, on 92 mm diameter plates) at room temperature (rT). For screening, all fungi were pre-cultured on identical plates for 7 days at 26°C ± 2°C in the dark. The selected fungi were pre-cultured for 14 days at 26°C ± 2°C in the dark on waterlogged chips of maple (*A. pseudoplatanus* L.), European birch (*B. pendula* Roth.), and poplar (*P. nigra* L.) that were sterilised at 105°C for 15 min then inoculated with two agar plugs (10 mm × 10 mm) after cooling.

Screening of zone line-forming fungi: The interaction between fungi was the focus of the screening. The fungi (Table 1) were co-cultivated in pairs on 1.5% malt extract agar plates at 26°C ± 2°C in the dark in five replicates for 14 days. For initiation, agar plugs (10 mm × 10 mm) were placed on the pre-cultures fungi pairs on the opposite edges of agar dishes. The growth, antagonism type and ZL-formation characteristics of the dual cultures were documented on days 1, 3, 7, 10, and 14 during each experiment and subsequently assessed. To eliminate slow growing fungi, fungal combinations that did not come into contact within 14 days were not further considered.

ZL formation: The fungal combinations were *L.t.*/*T.h.*I, *L.t.*/*T.v.* III, *P.e.*/*T.h.* I., and *P.e.*/*T.v.* III (for full names of the fungi see Table 1). In addition, *P.e.*/*L.t.* was included as a negative control. The following woods were tested: maple (*A. pseudoplatanus* L.; average oven-dry specific gravity 0.54 g cm^{-3}), European birch (*B. pendula* Roth.; 0.61 g cm^{-3}) and poplar (*P. nigra* L.; 0.34 g cm^{-3}). These woods have a low natural resistance against fungi (CEN 1994), weak patterning (low contrast between early and late wood), high availability and are known for susceptibility to ZL formation. Dimensions: 15 × 25 × 50 mm^3 (R × T × L) based on the standard method DIN EN 113 (CEN 1996) to facilitate evaluation of wood decomposition. The MC of the wood samples was adjusted to ca. 40% by impregnation with distilled water at rT after sterilisation, then fungal pre-cultures on chips of each wood type were spread on opposite transverse faces of triplicate wood samples, enabling the fungi to grow in the direction of the grain (Luthardt 1956; Jacobs et al. 2011; Wagenführ and Luthardt 2012). The inoculated wood samples were stored in polypropylene boxes (70 mm high, 140 mm wide and 200 mm long). A sterile filter with 0.2 µm pores (VWR, Darmstadt, Germany) was installed in each box to enable gas exchange, and water was added to a depth of 20 mm to maintain high MCs. The samples were incubated at 26°C in the dark for 8 weeks, then cultivation was stopped to minimise wood degradation. The mass loss (ML) of each wooden sample was measured and its mechanical properties were tested. To this purpose, the samples were dried at 103°C and weighed after reaching a constant weight. The process was repeated after incubation and carefully removing mycelia from their surfaces. The calculated %ML is based on the initial weight.

For data evaluation, the analysis of variance (ANOVA) followed by the F-test were applied.

For anatomical observations, two specimen blocks with a volume of ca. 1 cm^3 were carved out of a selected maple sample with a ZL formed by a *L.t.*/*T.h.* I dual culture. The blocks were soaked separately in distilled water at 23°C for 1 day. Then ca. 20 µm thin sections were cut by a cryomicrotome (Leica Frigomobil, Mannheim, Germany) and a steel knife (Leica) with wedge profile. For this, the blocks were fixed by a freezing agent (Tissue-Tek® O.C.T.™ Compound from SakuraFinetek, USA). The temperature was adjusted to –35°C to freeze the whole specimen block before cutting. The thin sections were dyed in a 0.5% (w/v) aqueous safranin solution and in a 0.5% (w/v) aqueous Astra blue solution, in each case for 1 min at 23°C. Then excess dye was rinsed off with distilled water three times (Gärtner and Schweingruber 2013). The resulting stained sections were embedded in glycerol gelatine (Kaiser's from Merck, Darmstadt, Germany) and examined under a light microscope (Zeiss Axio, Jena, Germany). A single-lens reflex camera (Canon EOS 600 D) with live view mode was available in combination with the software ImageJ (USA) for recording the images.

Table 1: Name, origin and abbreviations of the investigated fungi.

Name	Origin	Abbreviation
Armillaria mellea	DSM 2941	*A.m.*
Bjerkandera adusta	DSM 3375	*B.a.*
Coniophora puteana	DSM 3085	*C.p.*
Fomes fomentarius	CBS 311.8	*F.f.*
Ganoderma applanatum	(isolated)	*G.a.*
Gloeophyllum trabeum	DSM 3087	*G.t.*
Lentinus tigrinus	(isolated)	*L.t.*
Marasmius sp.	(isolated)	*M.s.*
Phellinus triviale	(isolated)	*P.t.*
Pleurotus eryngii	(isolated)	*P.e.*
Poria placenta	DSM 3088	*P.p.*
Trametes hirsuta	BT 2566	*T.h.* I
Trametes hirsuta	DSM 5072	*T.h.* II
Trametes hirsuta	DSM 5241	*T.h.* III
Trametes versicolor	FG 522	*T.v.* I
Trametes versicolor	DSM 1977	*T.v.* II
Trametes versicolor	DSM 3086	*T.v.* III

DSM, German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) (Germany); CBS, CBS-KNAW Fungal Biodiversity Centre (The Netherlands); BT, Collection of Institute of Environmental Biotechnology, Graz University of Technology (Austria); FG, Collection of Forest Botany Göttingen (Germany).

Results and discussion

Screening of zone line (ZL)-forming fungi

Figure 1 illustrates the basic types of interactions between fungi, i.e. overgrowth (Ov), contact inhibition (Ci) and antibiosis (An), as already described by Bertrand et al. (2013). However, in this study additional interaction combinations with and without pigmentation were observed, i.e. An with ZL formation (An + ZL), and An followed by Ci (An + Ci), whereby ZL and An + ZL were described by Rayner and Todd (1977). Directly at the contact point of the mycelia, some dual cultures formed a dark demarcation

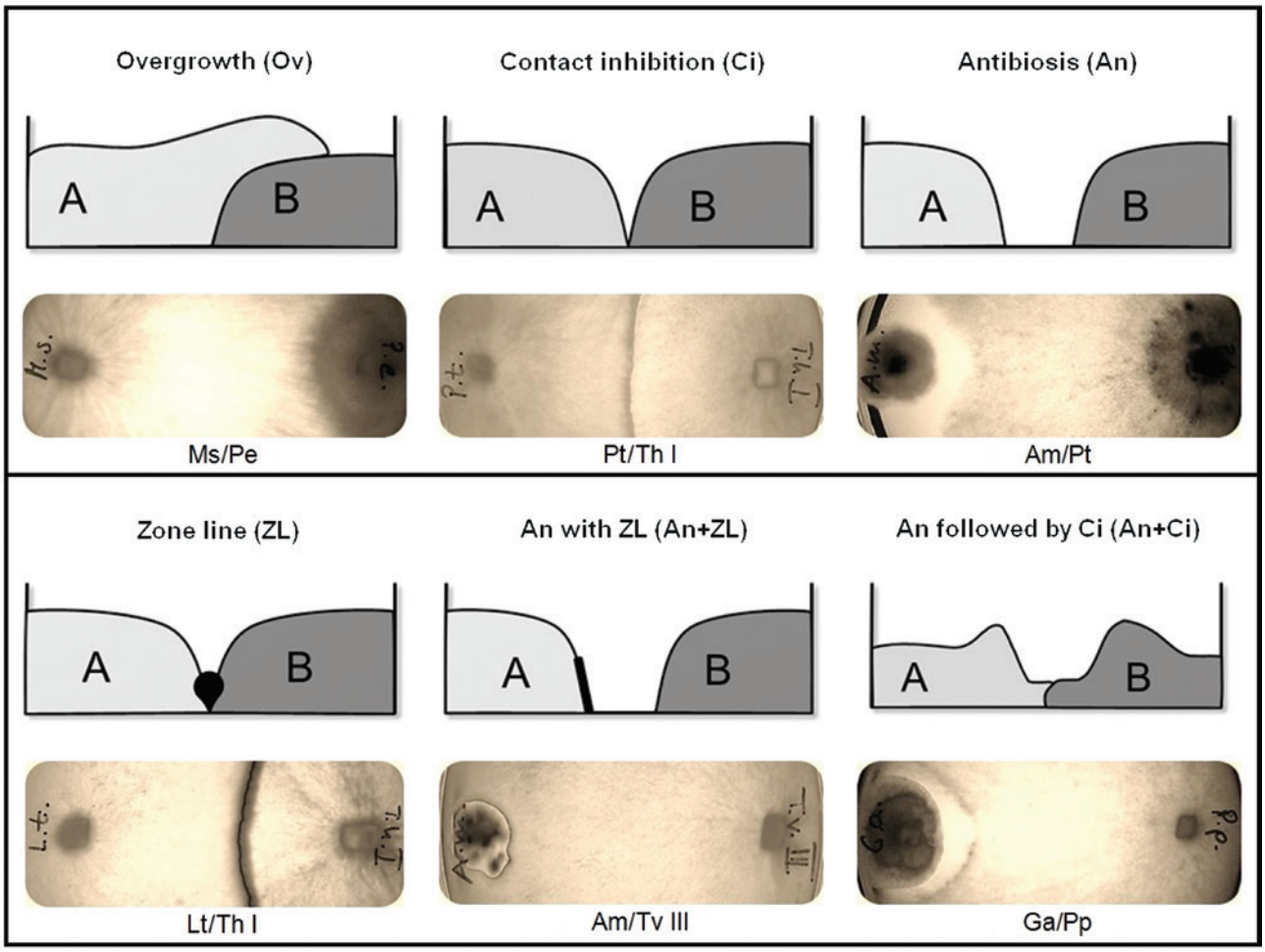

Figure 1: Schematic diagrams and photographs illustrating selected observed types of interactions between fungal cultures (A and B) on malt extract agar plates.
Schematic illustrations of Ov, Ci, An, and ZL are in the style of Bertrand et al. (2013). Full names of the fungi are presented in Table 1.

line (ZL), which was not only limited to the malt extract agar surface, but which was penetrating deep into the substrate. This type of antagonistic interaction was observed on 23 fungal combinations (Figure 2). The most frequently involved fungus was *L.t.* (in combination with 10 other fungi, including every tested *Trametes* strain). *P.e.* was involved in ZL formation in combination with seven other fungi. However, the combination of these two fungi did not result in ZL, instead *L.t.* overgrew *P.e.* (Figure 2), possibly because the melanin layer is destroyed by secreting laccases (Pohleven et al. 2008).

ZL formation is also influenced by specific moisture conditions (Tudor et al. 2012) or the nutrient profiles (Schmidt 2006). Interestingly, ZL formation was also observed before the fungi came into contact (An + ZL). The dual cultures *A.m.*/*T.v.* II and *A.m.*/*T.v.* III initially showed An type responses. However, after 10 days incubation the *T.v.* cultures began to form a dark line around their outermost mycelia. In the following days, the fungi did not grow further in these co-cultures, possibly because the demarcation of the *T.v.* cultures was inhibited. Changes of pH or MC can induce *T.v.* to produce ZLs without the presence of an antagonist (Schmidt 2006). However, in these cases, it can be assumed that *A.m.* released some inhibitory substance(s) or the changed environmental conditions disadvantaged *T.v.* II and *T.v.* III. An + Ci interaction was observed in the dual cultures *B.a.*/*G.t.* and *G.a.*/*P.p.*. In such cases, one of the fungi may exude substances that lead initially to An, but the antagonist degrades these substances and the growth of the mycelium is inhibited. *P.e.* formed ZLs in combination with strains *T.h.* I and *T.h.* II, but not in combination with *T.h.* III. This is probably due to differences in the genotypes (Schmidt 2006).

Table 2 shows frequencies of interaction types in the agar experiment. In almost a quarter of the tested dual

A \ B	Am	Ba	Cp	Ff	Ga	Gt	Lt	Ms	Pt	Pe	Pp	Th I	Th II	Th III	Tv I	Tv II	Tv III
Am	-	OvB	-	An	-	OvB	Ci	OvB	An	OvB	An	Ci	Ci	An	ZL	An+ZL	An+ZL
Ba		I	OvA	OvA	OvA	An+Ci	Ci	OvB	OvA	ZL	Ci	Ci	OvA	OvA	OvA	OvA	OvA
Cp			I	Ci	-	Ci	ZL	OvB	Ci	An	An	Ci	Ci	An	Ci	Ci	Ci
Ff				Ci	Ci	Ci	ZL	OvB	Ci	OvB	Ci	Ci	Ci	Ci	Ci	Ci	Ci
Ga					-	OvB	OvB	OvB	Ci	Ci	An+Ci	Ci	Ci	Ci	Ci	An	Ci
Gt						I	ZL	Ci	Ci	OvA	ZL	OvA	OvA	OvA	Ci	OvA	Ci
Lt							I	OvB	Ci	OvA	ZL	ZL	ZL	ZL	ZL	ZL	ZL
Ms								I	OvA	OvA	OvA	OvA	OvA	OvA	OvA	OvA	OvA
Pt									I	ZL	Ci	Ci	Ci	Ci	ZL	Ci	ZL
Pe										I	Ci	ZL	ZL	Ci	ZL	ZL	ZL
Pp											Ci	Ci	Ci	Ci	Ci	Ci	Ci
Th I												I	Ci	Ci	Ci	Ci	Ci
Th II													I	Ci	Ci	Ci	Ci
Th III														Ci	Ci	Ci	Ci
Tv I															I	Ci	Ci
Tv II																I	Ci
Tv III																	I

Figure 2: Interactions of dual cultures after 14 days (n = 5): (OvA), fungus A overgrows fungus B; (OvB), fungus B overgrows fungus A; (An), antibiosis; (Ci), contact inhibition; (ZL), zone line formation; (An + ZL), antibiosis with zone line formation; (An + Ci), antibiosis followed by contact inhibition; (I), indistinguishable; (–), not investigated.
The shaded boxes indicate dual cultures chosen for the wood experiment (four combinations with reproducible ZL and one selected as a negative control). Full names of the fungi are presented in Table 1.

Table 2: Frequencies of observed types of interactions of the tested basidiomycetes: (Ov), overgrowth; (An), antibiosis; (Ci), contact inhibition; (ZL), zone line; (An + ZL), antibiosis with zone line formation; (An + Ci), antibiosis followed by contact inhibition; (n.d.), not distiguishible.

Type of interaction	Frequency (%)
Ov	24
An	5
Ci	45
ZL	14
An + ZL	2
An + Ci	2
n.d.	8

cultures, one of the fungi was overgrown. *M.s.* appears to be the strongest of the tested organisms. This fungus overgrew in 15 cases of the tested 16 antagonists. Ci type was the most frequent type of interaction (45% of the combinations), and *T.h.* I. showed the highest frequency of Ci interactions (12 out of 16 in total). *A.m.* was most frequently involved in An interactions (four out of 14 in total). The rarest observed interactions were An + ZL and An + Ci (Table 2).

The brown rot (BR) fungi *C.p.*, *G.t.*, and *P.p.* were not yet described as ZL-forming species, but here ZLs were seen with *L.t.* This is not unexpected as *L.t.* always form ZLs in other fungal pairings too. Nevertheless, *G.t.*/*P.p.* formed ZLs on agar. However, the BR fungi were not further considered in the subsequent investigations due to the rapid depolymerization of carbohydrates already during the early stages of decay and the resulting strength loss of the samples.

P.t., *P.e.* and *T.h.* were not known previously as ZL-forming species, either. However, ZL formation is known in case of other fungi, such as of the genera *Phellinus* (e.g. Rypáček 1966; Becker 2010), *Pleurotus* (Robinson et al. 2007a; Becker 2010) and *Trametes* (e.g. Rypáček 1966; Weiß et al. 2000; Schmidt 2006; Robinson et al. 2007a; Becker 2010). Our study confirmed the ZL formation of the following fungi: *A.m.* (e.g. Lopez-Real 1975; Lopez-Real and Swift 1977; Schmidt 2006; Butin 2011), *B.a.* (Robinson et al. 2007a; Becker 2010), *F.f.* (e.g. Rypáček 1966; Schmidt 2006; Butin 2011), *L.t.* (e.g. Rypáček 1966), and *T.v.* (e.g. Weiß et al. 2000; Schmidt 2006; Robinson et al. 2007a; Becker 2010). On the other hand, the effectivity of *G.a.* with this regard could not be confirmed (Rypáček 1966).

Fungus		*A. pseudoplatanus*			*B. pendula*			*P. nigra*		
A	B	1	2	3	1	2	3	1	2	3
Lt	Pe	ZL	-	ZL	ZL	ZL	ZL	-	ZL	-
Lt	Th I	ZL	ZL	ZL	ZL	ZL	ZL	ZL	ZL	-
Lt	Tv III	-	ZL	-	-	-	ZL	-	-	-
Pe	Th I	-	-	-	-	-	-	-	-	-
Pe	Tv III	-	-	-	-	-	-	-	-	-

Figure 3: Presence (ZL) and absence (–) of zone line formation by indicated combinations of basidiomycetes in the triplicate maple, European birch, and poplar samples.
Full names of the fungi are presented in Table 1.

Figure 4: Zone line formed by a *Lt/Th* I dual culture in a maple sample.
Full names of the fungi are presented in Table 1.

ZL formation in hardwoods

In all dual cultures with *L.t.* (Figure 3), ZL formation could be confirmed, but not only in case of the combinations shown on malt extract agar experiments, but also for negative control combination *L.t./P.e.* (which yielded ZLs in all three hardwood species, but only in all three triplicates of European birch).

The *L.t./T.h.* I combination formed ZLs in eight out of nine samples, including all of the maple and European birch samples (Figure 3). However, *L.t.* grew significantly faster than *P.e.* under the test conditions, thus the melanin layer formed closer to the *P.e.* inoculation end and not in the middle. In contrast, *L.t.* and *T.h.* I grew at almost identical rates, so with this combination ZLs formed close to the sample middle (Figure 4).

As mentioned above, a demarcation line is a 3D layer, which can be seen in various places after cutting the wood either radially or tangentially (Figure 4). The ZL of maple depicted in Figure 4 was examined under a light microscope to obtain anatomical information. The RGB colour micrographs showed besides the red dyed wood structure, blue stained hyphae next to undyed naturally brown fillings of some lumens. It seems reasonable to assume that the dark fillings are melanised inflated hyphae. The micrographs of a transverse section and a radial section of the zone line area of a maple sample incubated with a *L.t./T.h.* I dual culture are presented in Figure 5. Here, a transverse section clearly shows the dark fillings in lumens (black arrow, 5b) and the white arrow shows hyphae. Dark fillings were described already in naturally formed ZLs in European birch (Jacobs et al. 2011) and sugar maple (Tudor et al. 2014). This black filling creates a barrier for other microorganisms (Rayner and Boddy 1988).

Half of the dual cultures successful on malt extract agar also developed clearly visible ZLs in wood. *L.t.* vs. *P.e.* served as negative control, which did not show ZL on malt extract agar but unexpectedly, developed the best ZLs on wood with a high reproducibility. Thus, ZL formation is both fungus and substrate specific. Essential factors are: relative growth rates and substrate preferences of the fungi (Robinson and Laks 2010; Tudor et al. 2013), relative amounts of cellulose, hemicelluloses, lignin and extractives in wood (Schmidt 2006), and the pH (Tudor et al. 2013). Figure 6 shows the mean ML data (7% to 22%) with each permutation of the hardwood species and the three dual fungal cultures yielding ZLs after 8 weeks incubation. Incubation of European birch with a *L.t./P.e.* dual culture and incubation of both maple and European birch samples with a *L.t./T.h.* I dual culture consistently resulted in ZL formation (mean ML: 7% to 18%). European birch suffered the lowest ML with an $ML_{max} < 10\%$. ML data of maple and poplar were higher in five cases out of six combinations of wood and dual cultures (Figure 6). WR fungi, such as *T.v.*, degrade lignin and carbohydrates simultaneously at similar rates (Schmidt 2006). Maple wood has a relatively high lignin content (nearly 26%), while poplar has high hemicellulose contents, nearly 32% (Wagenführ 2007), which may at least partly explain their higher degradation rates.

There is no direct interaction between type of hardwood and fungal combination regarding the ML data as evaluated by analysis of variance (ANOVA) followed by F-test. Furthermore, no significant influence of the fungal combination on the ML was detected. But the wood species are influential. Parameters just like durability or moisture-retaining ability have a higher influence on the decay and therefore on the ML. The ML data in Figure 6 illustrate the statistical calculations.

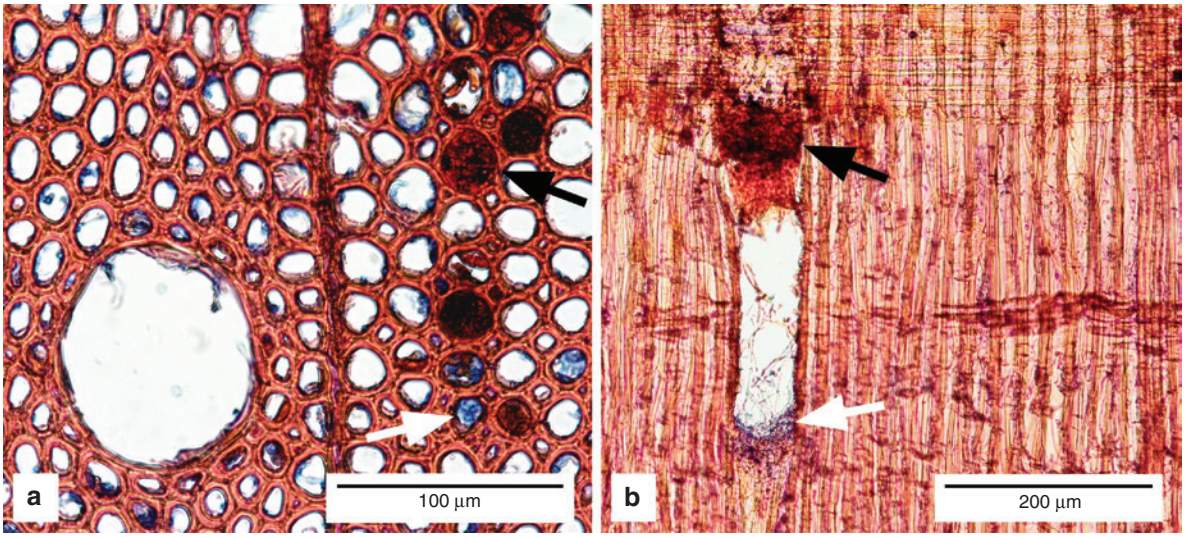

Figure 5: Micrographs of the zone line area of a maple sample incubated with a *Lt*/*Th* I dual culture. Transverse section (a) and radial section (b) with dark filled lumens (black arrows) and hyphae (white arrows).
Full names of the fungi are presented in Table 1.

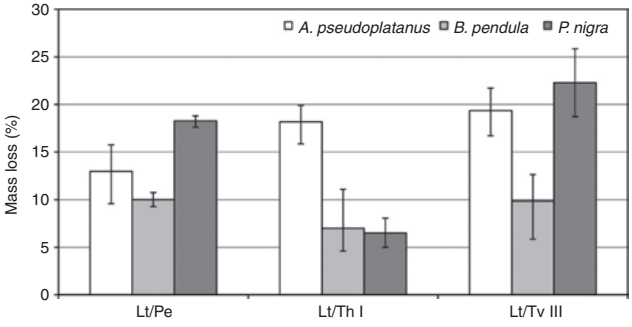

Figure 6: Mean mass losses during incubations with permutations of hardwood samples (maple, European birch and poplar) and dual cultures that consistently yielded zone lines after 8 weeks incubation. Whiskers represent maximal and minimal values (n = 3).
Full names of the fungi are presented in Table 1.

Conclusion

In addition to known basic types of interactions between fungi (overgrowth, contact inhibition, antibiosis, zone lines), several combinations of interaction types with and without pigmentation were observed on malt extract agar, such as antibiosis with zone line and antibiosis followed by contact inhibition. Furthermore, the ability of *Lentinus tigrinus* to form zone lines with many other fungi was observed including three brown rot fungi (*Coniophora puteana*, *Gloeophyllum trabeum* and *Poria placenta*). It was demonstrated that the ability of dual cultures to form zone lines is both fungus and substrate dependent, while the *Lentinus tigrinus* vs. *Trametes hirsuta* I combination formed reproducible zone lines on malt extract agar and in all of the maple and European birch samples; in popular sample this combination formed just two out of three samples zone lines. Incubating maple with a *Lentinus tigrinus* vs. *Trametes hirsuta* dual culture consistently led to formation of good quality zone lines in the middle of the samples, with a mean mass loss through degradation of the wood of nearly 18%. The results indicate that *Lentinus tigrinus* is the best zone line-forming basidiomycete (of those tested) in maple, European birch and poplar. To develop a mycological wood modification process that provides reproducible, good quality zone lines, further investigation is required. The main findings of this study should be verified with larger samples. Mechanical characteristics and the mass losses of the samples with good quality zone lines are helpful to estimate also their workability.

Acknowledgements: We would like to thank David Lenz, who performed numerous experiments during his work as student assistant and Dr. rer. silv. Björn Günther from the Faculty of Environmental Sciences of the Technische Universität Dresden for supporting the microscopy investigations.

References

Adams, D.H., Roth, L.F. (1967) Demarcation lines in paired cultures of *Fomes canjanderi* as a basis for detecting genetically distinct Mycelia. Can. J. Bot. 45:1583–1589.

AWPA E10-09 (2009). Standard Method of Testing Wood Preservatives by Laboratory Soil-Block Cultures; American Wood Protection Association, 04/01/2009.

Becker, K. (2010) Untersuchungen zur reproduzierbaren Zonenlinienbildung an Laubholz. Diploma Thesis, Technische Universität Dresden (in German).

Bertrand, S., Schumpp, O., Bohni, N., Bujard, A., Azzollini, A., Monod, M., Gindro, K., Wolfender, J.-L. (2013) Detection of metabolite induction in fungal co-cultures on solid media by high-throughput differential ultra-high pressure liquid chromatography–time-of-flight mass spectrometry fingerprinting. J. Chromatogr. A 1292:219–228

Butin, H. Krankheiten der Wald- und Parkbäume – Diagnose, Biologie, Bekämpfung. Verlag Eugen Ulmer, Stuttgart, 2011.

Campbell, A.H. (1933) Zone lines in plant tissues: I: the black lines formed by *Xylaria polymorpha* (Pers.) grev. in hardwood. Ann. Appl. Biol. 20:123–145.

Campbell, A.H. (1934) Zone lines in plant tissues: II. The black lines formed by *Armillaria mellea* (Vahl) quel. Ann. Appl. Biol. 21:1–22.

CEN (1994) DIN EN 350-2. Durability of Wood and Wood-Based Products – Natural Durability of Solid Wood – Part 2: Guide to the Natural Durability and Treatability of Selected Wood Species of Importance in Europe; German version EN 350-2:1994. European Committee for Standardization, Brussels, Belgium.

CEN (1996) DIN EN 113. Wood Preservatives – Method of Test for Determining the Protective Effectiveness Against Wood Destroying Basidiomycetes – Determination of the Toxic Values; German Version EN 113:1996. European Committee for Standardization, Brussels, Belgium.

Ellis, D.H., Griffiths, D.A. (1974) The location and analysis of melanins in the cell walls of some soil fungi. Can. J. Microbiol. 20:1379–1386.

Ellis, D.H., Griffiths, D.A. (1975) Melanin deposition in the hyphae of a species of *Phomopsis*. Can. J. Microbiol. 21:442–452.

Gärtner, H., Schweingruber, F.H. Microscopic preparation techniques for plant stem analysis. Dr. Kessel, Remagen-Oberwinter, 2013.

Harder, R. (1911) Über das Verhalten von Basidiomyceten und Ascomyceten in Mischkulturen. University Kiel 1911 (Dissertation; also: Naturwissenschaftliche Zeitschrift für Forst- und Landwirtschaft).

Hartig, R. Die Zersetzungserscheinungen des Holzes der Nadelbäume und der Eiche in Forstlicher, Botanischer und Chemischer Richtung. Springer, Berlin, 1878.

Hubert, E.E. (1924) The diagnosis of decay in wood. J. Agric. Res. 29:523.

Jacobs, K., Becker, K., Weiß, B., Scheiding, W. (2011) Untersuchungen zur Herstellung von Marmorholz durch Behandlung mit Pilzen. Holztechnologie 52:33–39.

Koch, K. (2014) Farb-Stoff. Furnier-Magazin (Supplement of Holz-Zentralblatt and HK) 18:62–65.

Lopez-Real, J.M. (1975) Formation of Pseudosclerotia ('Zone Lines') in Wood decayed by *Armillaria mellea* and *Stereum hirsutum* – I. Morphological aspects. Trans. Br. Mycol. Soc. 64, 465–471.

Lopez-Real, J.M., Swift, M.J. (1977) The formation of pseudosclerotia ('zone lines') in wood decayed by *Armillaria mellea* and *Stereum hisutum* – II. Formation in relation to the moisture content of the wood. Trans. Br. Mycol. Soc. 64:473–481.

Luthardt, W. (1956) Verfahren zur Veredlung von Holz. DE 000000946845 B/09.08.1956.

Michaelsen, H., Unger, J., Fischer, C.H. (1992) Blaugrüne Färbung an Intarsienhölzern des 16. und 18. Jahrhunderts. In: Restauro 1992, Georg D.W. Callwey GmbH & Co. KG, München, Germany, S17–S25 (in German).

Phillips, L.W. (1987) The nature of spalted wood: analysis of zone line formation between six white rot fungi. Master of Science Thesis, Brigham Young University.

Pohleven, F., Vidic, I., Tavzes, Č. (2008) Degradation of melanin and biocides by ligninolytic fungi. Wood Science for Conservation of Cultural Heritage – Proceedings of the International Conference held by COST Action IE0601 (Braga – Portugal, 5–7 November 2008):129–134

Rayner, A.D.M., Boddy, L. Fungal Decomposition of Wood – its Biology and Ecology. Wiley, Chichester, 1988.

Rayner, A.D.M., Todd, N.K. (1977) Intraspecific antagonism in natural populations of wood-decaying basidiomycetes. J. Gen. Microbiol. 103:85–90.

Robinson, S.C. (2012) Developing fungal pigments for "painting" vascular plants. Appl. Microbiol. Biotechnol. 93:1389–1394.

Robinson, S.C., Tudor, D., Hipson, S., Snider, H., Ng, S., Korshikov, E., Cooper, P.A. (2013) Methods of inoculating *Acer spp*., *Populus tremuloides*, and *Fagus grandifolia* logs for commercial spalting applications. J. Wood Sci. 59:351–357.

Robinson, S.C., Laks, P.E. (2010) Wood species and culture age affect zone line production of *Xylaria polymorpha*. Open Mycol. J. 4:18–21.

Robinson, S.C., Richter, D.L., Laks, P.E. (2007a) Colonization of sugar maple by spalting fungi. Forest Prod. J. 57:24–32.

Robinson, S.C., Laks, P.E., Richter, D.L., Pickens J.B. (2007b) Evaluating loss of machinability in spalted sugar maple. Forest Prod. J. 2007:33–37.

Robinson, S.C., Laks, P.E., Turnquist E.J. (2009a) A method for digital color analysis of spalted wood using Scion image software. Materials 2:62–75.

Robinson, S.C., Richter, D.L., Laks, P.E. (2009b) Effects of substrate on laboratory spalting of sugar maple. Holzforschung. Band 63:491–495.

Robinson, S.C., Laks, P.E., Richter, D.L. (2011a) Stimulating spalting in sugar maple using sub-lethal doses of copper. Eur. J. Wood Prod 69:527–532.

Robinson, S.C., Tudor, D., Cooper, P.A. (2011b) Wood preference of spalting fungi in urban hardwood spedies. Intern. Biodet. Biodegr. 65:1145–1149.

Robinson, S.C., Tudor, D., Cooper, P.A. (2012) Promoting fungal pigment formation in wood by utilizing a modified decay jar method. Wood Sci. Technol. 46:841–849.

Robinson, S.C., Michaelsen, H., Robinson, J.C. Spalted Wood: The History, Science, and Art of a Unique Material. Schiffer Publishing Ltd., Atglen, USA, 2016, pp. 287.

Rypáček, V. Biologie holzzerstörender Pilze. Fischer, Jena, 1966.

Schmidt, O. Wood and Tree Fungi – Biology, Damage, Protection, and Use. Springer, Berlin Heidelberg, 2006.

Schwarze, F.W.M.R., Engels, J., Mattheck, C. Holzzersetzende Pilze in Bäumen – Strategien der Holzzersetzung. Rombach. Freiburg im Breisgau, 1999.
Tudor, D., Robinson, S.C., Cooper, P.A. (2012) The influence of moisture content variation on fungal pigment formation in spalted wood. AMB Expr. 2:69.
Tudor, D., Robinson, S.C., Cooper, P.A. (2013) The influence of pH on pigment formation by lignicolous fungi. Intern. Biodeter. Biodegr. 80:22–28.
Tudor, D., Robinson, S.C., Sage, T.L., Krigstin, S., Cooper, P.A. (2014) Microscopic investigation on fungal pigment formation and its morphology in wood substrates. Open Mycol. J. 8:174–186.
Wagenführ, R. Holzatlas. Hanser, München, 2007.
Wagenführ, A., Luthardt, H. (2012) Review: Myko-Holz: eine (fast) vergessene deutsche Erfindung. Holztechnologie 53:48–50 (in German).
Wagenführ, A., Scholz, F. Taschenbuch der Holztechnik. Hanser, München, 2012.
Weiß, B., Wagenführ, A., Kruse, K. Beschreibung und Bestimmung von Bauholzpilzen. DRW, Leinfelden-Echterdingen, 2000.
Witzany, G. Biocommunication and Natural Genome Editing. Springer, Dordrecht, Netherlands, 2010.