Effects of catechol treatment on fungal melanin stimulation for spalting

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Abstract

The spalting process caused by fungi has a dramatic visual effect through unique random patterns of zone lines and coloration, and the resulting spalted wood is of interest due to its increased economic and artistic value. The most common pigment found in spalted wood is the dark colored melanin, produced in consecutive granule depositions in the lumens of the wood cells, to form distinctive zone lines. Fungal melanins on wood substrate are complex polymers synthesized from phenolic derivatives, and catechol is one of the melanin precursors for many fungi. To investigate whether fungal melanin formation can be stimulated by addition of catechol to the substrate we treated *Acer saccharum* and *Fagus grandifolia* samples with catechol at six concentration levels plus a control (no addition). Samples were inoculated in monocultures of *Trametes versicolor, Xylaria polymorpha* and *Inonotus hispidus*. The results indicate that *Trametes versicolor* produced zone lines in beech at lower concentrations of catechol, while *Inonotus hispidus* produced pigmentation in parenchyma cells at higher concentrations of catechol in both beech and sugar maple. Ascomycete *Xylaria polymorpha* showed no reaction to catechol for melanin formation, but the increase of mass loss could indicate a stimulation of its metabolism. The results indicate that catechol could be used for fungal pigment stimulation in wood.

Introduction

Spalting is a specific biological process of wood biodeterioration, characterized by fungal pigmentation in wood substrate as a result of environmental and microbial stress; it is characterized by zone demarcation lines and colorations (Fig.1). The most well-known pigment in spalting is melanin, a black phenolic biopolymer, non vital for fungal growth, but known to enhance virulence in pathogenicity and protection for fungal hyphae (Wheeler and Bell 1985, Butler and Day 1998, Henson *et al.*1999, Pearce 1991, Campbell 1934). The polymer is very resistant to most forms of degradation (Piattelli et al 1965, Selvakumar et al 2008), and has a high capacity to bind with metal ions increasing fungal resistance to toxic levels of metals (Fogarty and Tobin 1996). Butler et al (2001)

noted that melanins are able to absorb sound and electromagnetic energy, all wavelengths of light, from visible light to gamma rays, X-rays, ultraviolet and infrared light, transferring the energy at the cell level. Those properties define melanin as the ultimate survival kit for fungi.

Spalted wood has considerable artistic and economic value (Donovan and Nicholls 2003), and is especially appreciated in for musical instruments, fine furniture, flooring, and other artistic objects (Fig 2), and is fairly rare on the market. Our research interest is to investigate fungal pigment formation within the decay process for direct application to spalted wood production. Once the defense mechanism is triggered by various conditions of environmental stress, consecutive granules of melanin are deposited in the lumens of the wood cells to form distinctive zone lines (Bell and Wheeler 1986). The complete establishment of melanized zone lines can take from six month to three years even in highly susceptible species like *Acer saccharum.*

Fig.1 Pattern of zone lines and coloration characteristic to spalted wood

Fig. 2 Examples of wooden objects from spalted wood produced in the Applied Mycology Laboratory, University of Toronto: a- pen turned from spalted maple; b- bowl made from spalted maple; c- birch panel spalted on the surface.

Stimulation of zone line formation with minimal intervention, in shorter periods of time, with minimum strength loss of the wood substrate is necessary for developing a profitable production process. Also, promotion of spalting formation in underutilized wood species (e.g. *Fagus grandifolia*) is of great interest. Introduction of synthetic melanin precursors in wood substrate could deliver the requirements for a productive spalting process in non-sterile conditions. However, the identification of a common precursor for a diverse group of fungi is a rather difficult task.

Fungal hyphae produce melanin in the cell walls as well, and most abundant, as extracellular granular melanin (Fig.3), through utilization of phenol compounds secreted by fungi from the substrate (Bell and Wheeler 1986). The two types of melanin have slightly different properties, and most likely are produced by different biosynthesis pathways (Fogarty and Tobin 1996). It is also known that ascomycetes fungi synthesized predominantly melanin from 1,8 dihydroxynaphthalene (DHN), while basidiomycetes fungi usually produce catechol or y-glutaminyl-4-hydroxybenzene (GHB) melanin (Turner 1971, Wheeler and Bell 1985).). Catechol melanins are the most common black pigments in nature (Nicolaus et al 1964). It was demonstrated that catechol is an activator of tyrosinase, an enzyme involved in the last step of melanin production, and is presence restores pigment formation in fungal albino mutants (Duckworth and Coleman 1969, Wheeler et al 1978, Wheeler et al 1976).

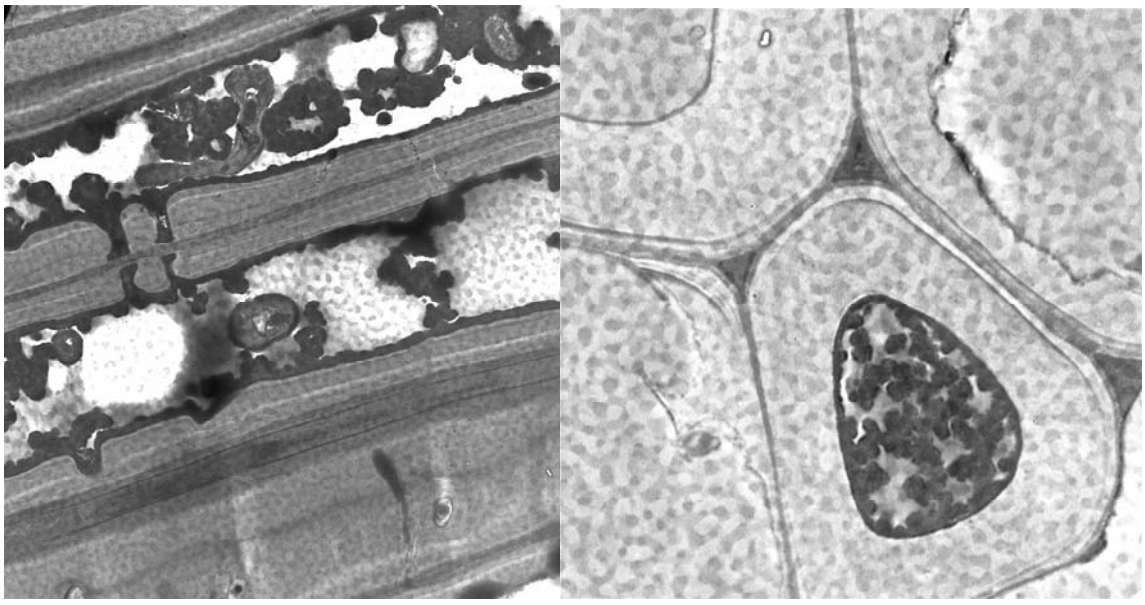


Fig.3 Electron imaging of wood cells with pigment deposits: two distinctive types of melanin formation: small granules deposited along the cell wall, possible bounded by wood cell polysaccharide, and bigger free granule formed in vesicle inside wood cells, probable associated with sclerotial mycelium.

Experimental Methods

Wood species tested were harvested within southern Ontario, based on their ability to spalt wood: Sugar maple (*Acer saccharum* Marshall) which develops extensive fungal pigmentation and zone lines, and beech (*Fagus grandifolia* L.) which more rarely spalts. The average oven-dry specific gravity of the samples tested was SG=0.68 for sugar maple and SG=0.74 for beech.

Three fungi were selected based on their pigment production ability: the ascomycete Xylaria polymorpha (Pers.) Grev., and two basidiomycete fungi, Trametes versicolor (L.) Lloyd and Inonotus hispidus (Bull.) P. Karst., (1879). Fungi used in experiment were grown on 95x15 mm Petri dishes with 2% malt extract agar at room temperature for two weeks prior to inoculation.

To test the influence of catechol on fungal growth, we prepared petri dishes with 1ppm, 7ppm, 10ppm, 70ppm, 100ppm, and 700ppm catechol in 1% agar. Fungi were inoculated in petri dishes and incubated at 21C. Measurements of fungal growth and pigmentation were recorded every two days in the first week and weekly for up to one month.

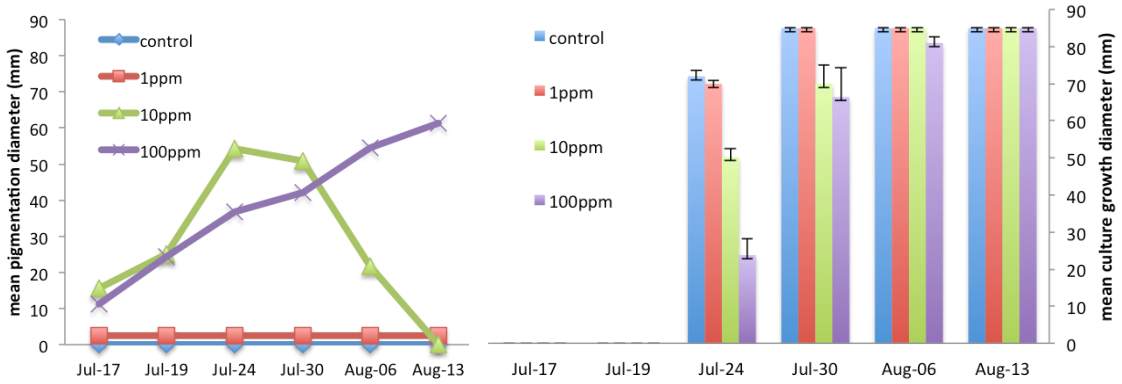
To test the influence of catechol on fungal pigment production in wood substrate, wood blocks were inoculated with fungi following a modified protocol for decay jar test, replacing soil with vermiculite, as outlined in Robinson et al (2009b), to avoid eventual influence of soil substrates on pigment formation. Sugar maple and beech 14 mm cubes, nine replicas per set, were weighed and then treated under vacuum for one hour with catechol solution adjusted for 1ppm, 7ppm, 10ppm, 70ppm, 100ppm, and 700ppm retention. One additional set was added for controls. After treatment, wood blocks were kept overnight at 40 C for conditioning, followed by steam sterilization and inoculation in sterile jars containing vermiculite. Based on their virulence *Trametes versicolor* jars were incubated for eight weeks, *Xylaria polymorpha* jars were incubated for ten weeks, and *Inonotus hipidus* jars were incubated for 12 weeks. At the end of the incubation, blocks were evaluated for decay (weight loss) and external/internal spalting amounts.

For pigment assessment, Wood samples were scanned with Epson WorkForce 500 scanner at 2400 dpi on one external side with the most pigment occurrence, and on an internal face, after the blocks were cut in half to expose a radial section. External and internal pigment evaluation was performed with Scion Image software, following the protocol described in Robinson et al. 2009a. Data were analyzed with a one-way ANOVA followed by Tukey HSD using SAS, version 9.2.

Results and Discussion

Fungal reaction to catechol in agar substrate

Trametes versicolor inoculated in 10 ppm catechol concentration reached maximum level of pigmentation after one week the colonies, followed by melanin degradation, while colonies exposed to 100 ppm catechol continue to maintain a lighter but extended pigmentation (Fig 4a and Fig 5). The fungus was growth inhibited by catechol up to two weeks at higher and medium levels of concentration (Fig. 4b). Control and 1ppm samples produced no pigmentation at any time. Similar behavior of the fungus to catechol was previously reported by Hart & Hillis (1974) and Taylor et al (1988). They observed an inhibition of growth when catechol was applied during the early stages of growth.



(a) (b)

Fig.4 The effect of catechol on pigmentation (a) and growth (b) of *Trametes versicolor* culture in 1% agar. Data shown are the means of five replicates

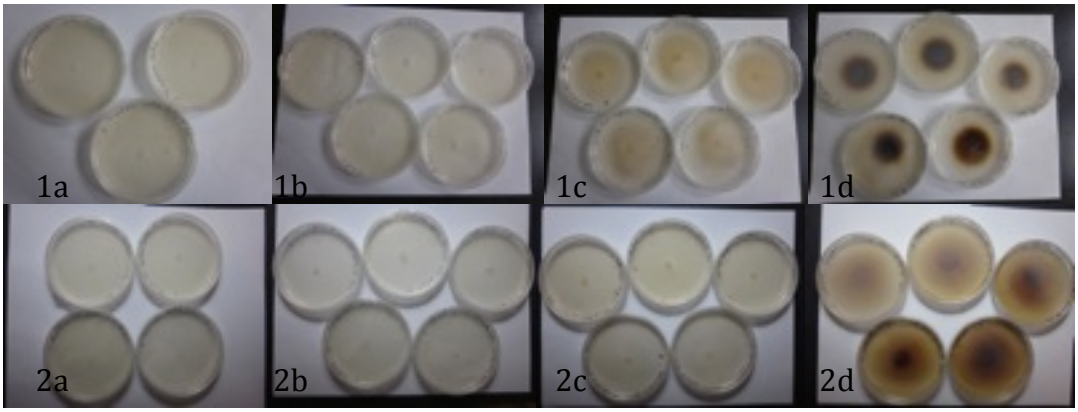
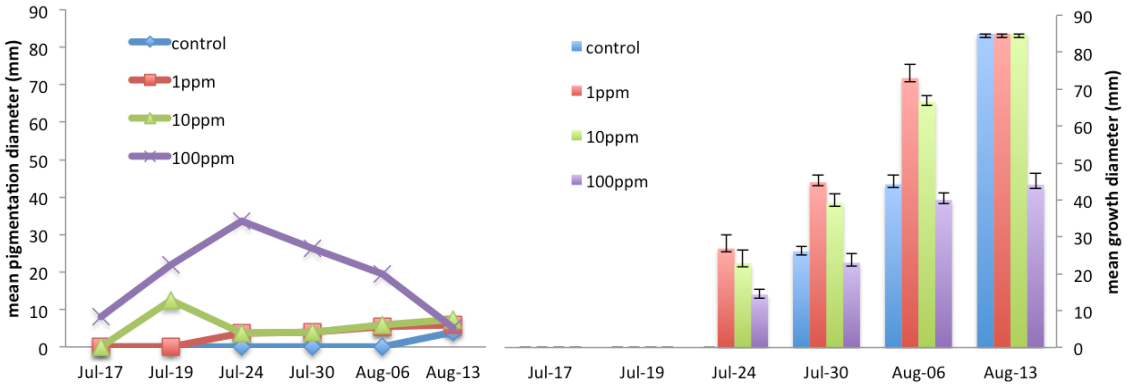


Fig.5 *Trametes versicolor* colonies in 1% agar: 1a,b,c,d, after one week from inoculation; 2a,b,c,d, after one month from inoculation; a-control, b-1ppm catechol, c-10ppm catechol, d-100ppm catechol on agar

Xylaria polymorpha reached maximum pigmentation also at one week after inoculation followed shortly by complete degradation of melanin (Fig. 6a), and was stimulated in growth mostly by smaller amounts of catechol (1 and 10 ppm, Figure 6b). Although the colonies of *X. polymorpha* inoculated in 100ppm catechol in agar started to develop mycelium before control samples, overall they had the slowest growth rate; a maximum pigmentation was registered in the second week. (Fig.6a and Fig.7).



(a) (b)

Fig.6 The effect of catechol on pigmentation (a) and growth (b) of *Xylaria polymorpha* culture in 1% agar. Data shown are the means of five replicates

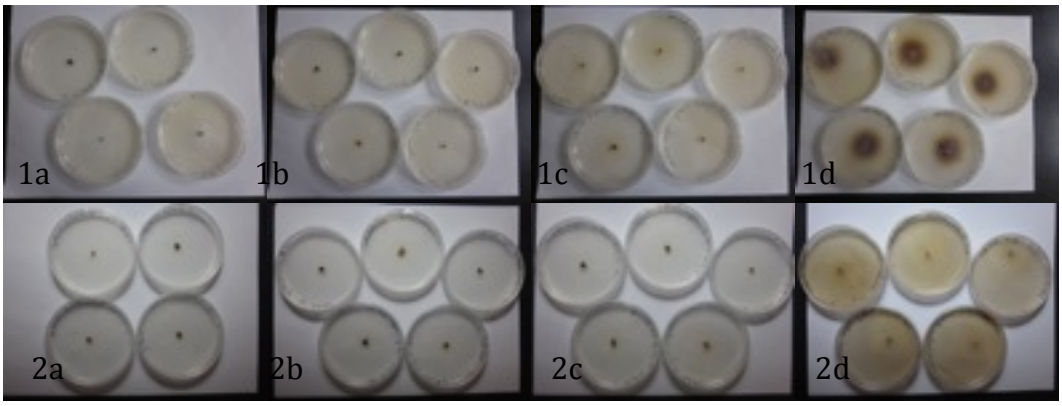
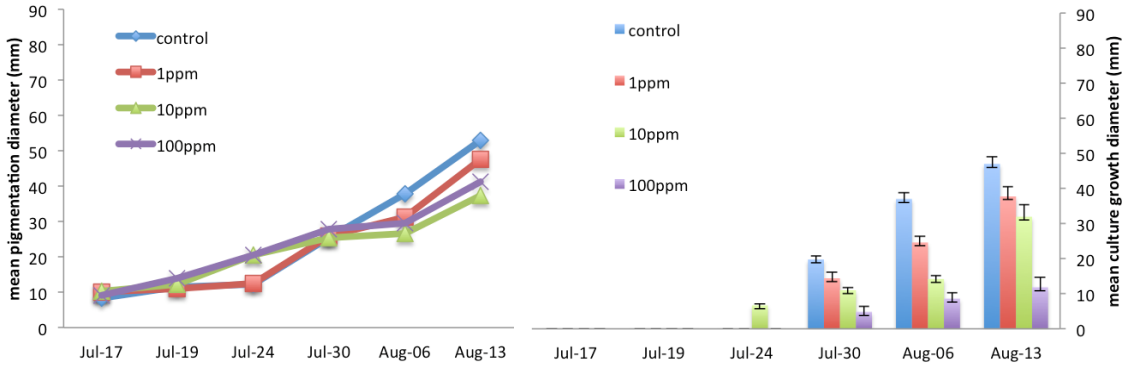


Fig.7 *Xylaria polymorpha* colonies in 1% agar: 1a,b,c,d, after one week from inoculation; 2a,b,c,d, after one month from inoculation; a-control, b-1ppm catechol, c-10ppm catechol, d-100ppm catechol on agar

Inonotus hispidus is not affected by catechol regarding the surface pigmented, but the density of pigmentation is the highest at 100ppm concentration, accompanied by the highest inhibition of fungal growth (Fig.8a,b and Fig.9)



(a) (b)

Fig.8 The effect of catechol on pigmentation and growth of *Inonotus hispidus* culture in 1% agar. Data shown are the means of five replicates

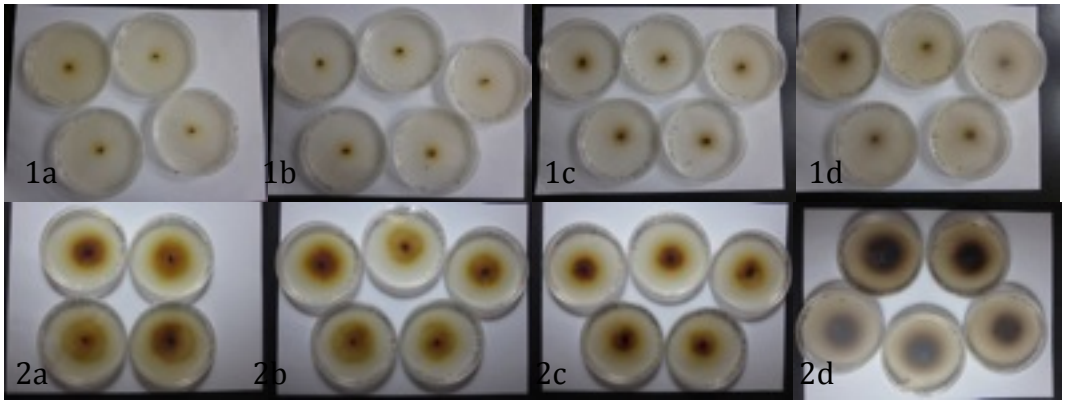


Fig. 9 *Inonotus hispidus* colonies in 1% agar: 1a,b,c,d, after one week from inoculation; 2a,b,c,d, after one month from inoculation; a-control, b-1ppm catechol, c-10ppm catechol, d-100ppm catechol on agar

Pigment formation in wood substrate

To test the influence of catechol on fungal pigment formation in wood samples, a one-way ANOVA was run, followed by Tukey's HSD at $\alpha = 0.05$, with fungal inoculation and induced catechol retentions as independent variables, for each wood species.

Internal pigment production (ip) and internal zone lines formation (izl) were significantly higher only for beech samples inoculated with *T. versicolor* and treated for 100ppm catechol retention ($P<0.0001$) (Fig10). There was no effect in sugar maple with any of the fungi tested.

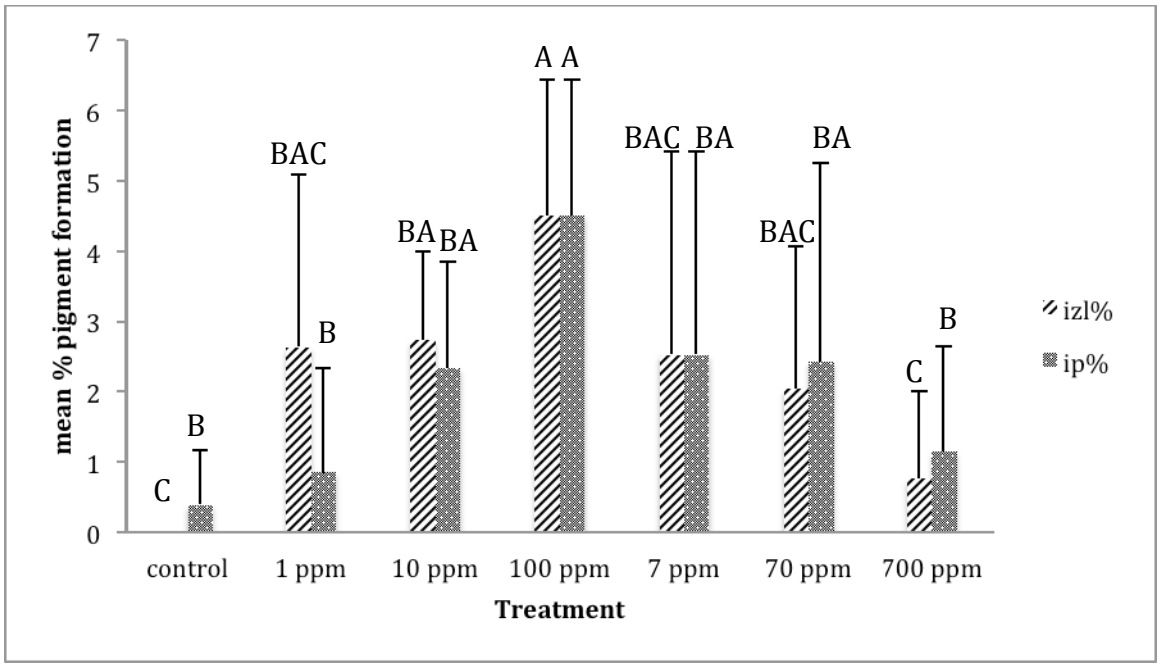


Fig.10 Internal pigmentation and zone line formation in beech. Data shown are the means of nine replicates. The graph shows means of pigmentation surface in percentages. Bars with different letters represent significantly different means.

As a general observation, external pigmentation was not produced by *T. versicolor* in the control samples (no catechol added) and the lowest addition (1ppm) concentration. Notable pigmentation gradually developed with increased catechol concentration (Fig 11).

Fig.11 Representative samples of pigmentation produced by *Trametes versicolor* in beech: a- external pigmentation in control sample; b - external pigmentation in beech treated for 700ppm catechol retention; c- internal zone line in beech treated for 100ppm catechol retention.

Xylaria polymorpha produced no internal zone lines in beech or sugar maple, although some pigment formation was evidenced at 70ppm in beech and 7ppm and 10ppm in sugar maple. An interesting behavior was noted at 700ppm, where the fungus in sugar maple pigmented the wood ray cells considerably. On the exterior of those samples, the zone lines were well defined (Fig12). Some

reactions were registered when samples were treated with catechol, and the pigment was present internally in many samples but not enough to be quantified.

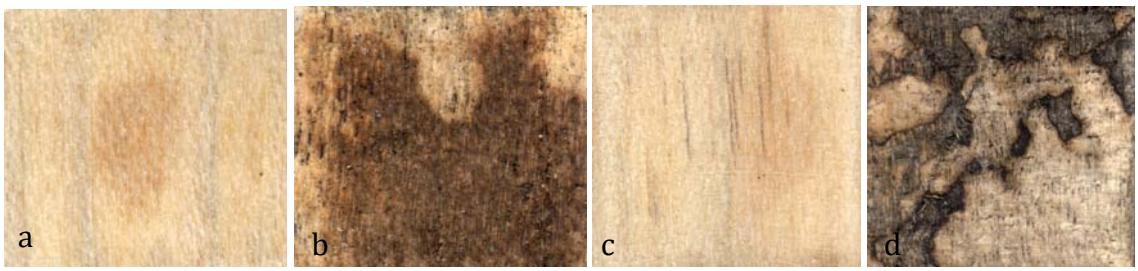


Fig.12 Representative samples of pigmentation produced by *Xylaria polymorpha* in sugar maple: a,b - internal and external pigmentation in control sample respectively; c,d- internal and external pigmentation in sugar maple treated for 700ppm catechol retention.

Inonotus hispidus is known to produce a yellow-brown pigment, hispidin, probably by phenylalanine oxidation (Perrin and Towers 1973). When exposed to catechol there was no significant increase of pigment formation in either wood species, except that in both agar and wood substrate tests, at 700ppm catechol exposure, the fungus synthesized a darker than usual pigmentation (Fig.9 and Fig13). Based on weight loss percentage in wood sample, fungal activity was inhibited by catechol at all levels of concentration with approximately 50% lower mass loss (56% for sugar maple and 47% beech) compared with control samples (Table1).

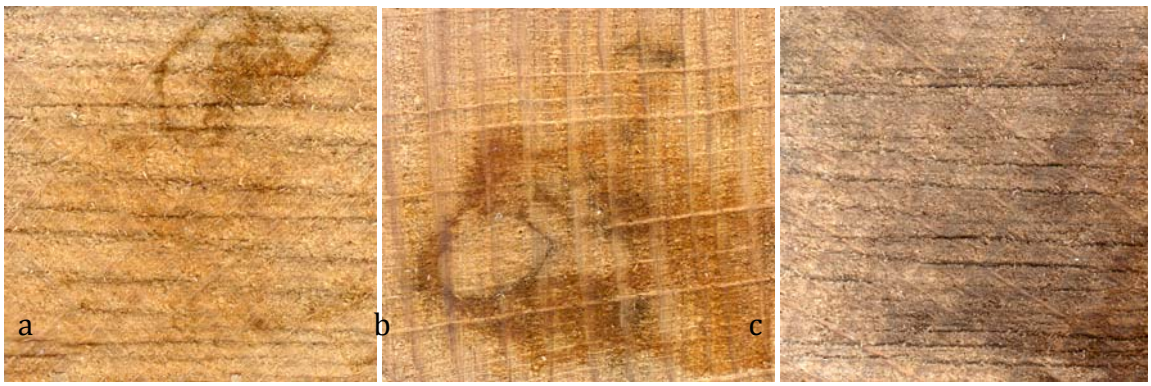


Fig.13 Representative samples of pigmentation produced by *Inonotus hispidus* in beech: a-control samples; b- beech with 7ppm catechol; c-beech with 700ppm catechol.

Table 1 Summary of data for untransformed weight loss for all tested fungi in both sugar maple and beech

Treatment	**Mean % weigh loss**					
	%			St.Dev.		
	Trametes v.	*Xylaria p.*	*Inonotus h.*	*Trametes v.*	*Xylaria p.*	*Inonotus h.*
Sugar maple						
control	11.3	1.9	3.3	2.2	2.3	1.3
1ppm	10.9	7.1	1.8	1.9	0.7	1.4
7ppm	5.2	4.5	2.2	2.0	2.2	1.9
10ppm	7.2	4.4	1.8	3.5	2.1	2.9
70ppm	8.0	6.0	1.3	3.4	2.7	1.5
100ppm	9.7	6.1	2.3	5.9	1.1	2.1
700ppm	3.4	5.5	1.8	5.2	4.0	1.9
Beech						
control	13.1	6.4	2.9	8.1	2.0	1.0
1ppm	8.0	7.1	1.5	7.9	1.9	1.0
7ppm	11.3	4.3	0.9	9.1	3.2	1.6
10ppm	11.6	5.7	1.9	6.0	1.9	1.6
70ppm	13.3	5.7	0.8	6.6	3.2	1.1
100ppm	13.2	6.9	1.7	6.8	2.0	1.2
700ppm	9.7	6.6	1.3	6.3	5.2	1.2

Conclusions

This research indicates that fungal pigment formation could be stimulated in beech inoculated with *Trametes versicolor* in monoculture by phenolic enhancement with catechol treatment. The growth inhibition at early stages of fungal development is an advantage, since the wood degradation is reduced. All fungi tested showed an ability to utilize catechol for melanin biosynthesis. The scattered pigment initiation, formed within the wood cells in fungal monoculture, are an indication of the potential engagement of melanin in zone lines configuration as a defense mechanism against antagonistic reactions. Further research, should test the reaction to catechol in fungal activity in dual inoculation.

Acknowledgments

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