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Screening fungi tolerant to Western red cedar (*Thuja plicata* Donn) extractives. Part 2. Development of a feeder strip assay

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Abstract

Western red cedar (*Thuja plicata* Donn) is a naturally durable softwood species native to British Columbia in Canada, and Washington, Oregon and California in the USA. Untreated *T. plicata* products are usually durable because of the presence of extractives with anti-microbial activity. However, there are extractive tolerant fungi that can attack *T. plicata*. To screen for extractive-tolerant species we developed a *T. plicata* feeder strip assay. When the feeder strips were placed on malt extract agar, extractives from the wood accumulated in the media and strongly inhibited growth of non-tolerant fungal strains. Extractives remaining in feeder strips following incubation on media were characterized. Of the many compounds leached out, γ- and β-thujaplicin, β-thujaplicinol, plicatic acid and thujic acid were quantified. The growth of selected fungal strains was not affected by plicatic acid; however, it was inhibited to different degrees by β- and γ-thujaplicin. *Pachnocybe ferruginea* was extractive-tolerant and may play an important role in the initial stages of degradation of *T. plicata* products.

Keywords: decks; extractives; pioneer fungi; β-thujaplicin; γ-thujaplicin; Western red cedar.

Introduction

Western red cedar (*Thuja plicata* Donn) is a naturally durable softwood species native to British Columbia, Canada, and Washington, Oregon and California in the USA. Its heartwood contains higher concentrations of aromatic and polyphenolic extractives than other softwood species (Barton and MacDonald 1971). Several of these compounds exhibit strong antimicrobial activity (Inamori et al. 1999, 2000; Arima et al. 2003; Morita et al. 2004) and may protect standing trees (Van der Kamp 1986; DeBell et al. 1997). Products made of *T. plicata* are known to be durable. As a result, Western red cedar is well suited for exterior residential applications and accounts for a significant proportion of Canada's forest products industry (Gonzalez 2004).

T. plicata products can still fail in service due to a) depletion of extractives caused by weathering or b) colonization by extractive-tolerant fungal species. Weathering involves photodegradation of extractives by UV radiation in sunlight and leaching out of degradation products from the surfaces (Coombs and Trust 1973; Johnson and Cserjesi 1980; Chedgy et al. 2005). There are pioneer fungi that preferentially attack weathered woods. Van der Kamp (1975) and Lim et al. (2005) isolated pioneer fungi from standing *T. plicata* trees and products in service. Such species do not decay wood, but may detoxify fungicidal extractives, facilitating subsequent colonization by decay fungi that are less tolerant to extractives (Jin et al. 1988).

To date only a few pioneer fungi have been isolated and characterized. In this work, a method is described for screening *T. plicata* extractive-tolerant fungi. Lim et al. (2005) performed such screening experiments in agar media mixed with extractives or pure compounds at different concentrations. Synthesis or isolation of total or specific extractive compounds is costly. A single compound may not reflect the synergistic effect of several extractives. Given this, we developed feeder strips (*T. plicata* FS) to detect decay and pioneer fungi that are tolerant to a mixture of naturally occurring *T. plicata* extractives. Results for fungal growth with *T. plicata* FS were compared to data obtained with single extractives.

Materials and methods

Fungal isolation and identification

T. plicata boards were collected from house decks and experimental test sites located in Vancouver, B.C., Canada. The service life of decks ranged from 20 to 100 years. We sampled 12 boards from deck A (100 years), five boards from deck B (20 years) and seven boards from deck C (25 years). Two sections of approximately 2 cm in thickness were cut from each board using a circular saw. Fungi were isolated within and near decay pockets as described by Lim et al. (2005). A 1% malt extract agar (MEA) was used for isolating general fungal flora and 1% MEA with benomyl (BMEA) was used for basidiomycete fungi (Clubbe and Levy 1977). Isolates were grouped by macro- and micro-morphological characteristics using taxonomic guides and standard procedures (Nobles 1965; Stalpers 1978; Wang and Zabel 1990). This initial identification was complemented by molecular techniques.

DNA was extracted from mycelia and the internal transcribed spacer (ITS) region was amplified using the primers ITS5 and ITS4 (Lim et al. 2005; White et al. 1990). PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen Inc., Mississauga, ON, Canada). Sequencing was performed on an ABI

3700 automated sequencer (Perkin-Elmer Inc., Foster City, CA, USA) at the DNA Synthesis and Sequencing Facility, Macrogen (Seoul, Korea). All nucleotide sequences presented in this work have been deposited at GenBank and their accession numbers are shown in Table 1.

Inhibition of fungal growth by *T. plicata* FS

T. plicata FS of approximately 5 cm × 3 cm × 0.2 cm in size were obtained from the outer heartwood of a sound 80-year-old *T. plicata* tree harvested at UBC Malcolm Knapp Research Forest, Maple Ridge, British Columbia. All wood samples originated from a single longitudinal axis parallel to the growth rings. Strips were sequentially numbered relative to their position. Samples were immediately placed into individual sealable bags, labeled and stored at -20°C until further use to minimize volatilization of extractives. The FS used in this study were sterilized by γ-radiation to prevent alteration of extractives that might occur during sterilization by alternative methods such as autoclaving. To measure fungal growth, an agar plug (5 mm in diameter) from a freshly grown fungal isolate on MEA was transferred onto 1% MEA with a *T. plicata* FS. The inoculum and the *T. plicata* FS were placed on opposite sites of the MEA plate. Fungi were also grown on 1% MEA without FS (control). All plates (three replicates per isolate) were incubated at 20°C in the dark to prevent extractive photodegradation. Fungal growth for each culture was measured every three days by taking two perpendicular measurements from the inoculum to the maximum and minimum growing edges of the colony. The two measurements were averaged. The average growth rate (mm day^{-1}) was calculated after 21 days. Growth inhibition tests were performed on 1% MEA supplemented with individual extractives from *T. plicata*. The added concentration for each extractive compound was calculated from the *T. plicata* FS experiment (see below).

Extractive analysis of *T. plicata* FS

T. plicata FS were placed onto 1% MEA and we determined the amount of extractives that leached out of the strips and accumulated in the media. (–)-Plicatic acid, γ-thujaplicin, β-thujaplicin, β-thujaplicinol, and thujic acid were extracted, separated and quantified (as μg g^{-1} based on dry weight) by reverse-phase (RP)-HPLC equipped with a UV detector as described by Chedgy et al. (2007). The compounds quantified are known to have antimicrobial activity and occur in high amounts in *T. plicata* heartwood. The strips were cut into two equal sections and weighed. One section was oven-dried at 105°C for 24 h to calculate the dry weight. The second section was finely ground and subjected to chemical analysis.

This experiment followed a completely randomized design (CRD) with three treatments: *T. plicata* FS (1) frozen at -20°C (control), (2) placed in empty plates and stored at 20°C for 21 days; and (3) stored on 1% MEA at 20°C for 21 days ($k = 3$). Six replicates were prepared in each case and the mean concentration of extractives was then calculated. Other details of the statistics, including the Tukey (1949) test used, are reported by Chedgy et al. (2007). Data presented in this paper as a percentage are by weight on oven-dry basis.

Results and discussion

Fungal identification and extractive resistance test

A total of 242 fungal isolates were recovered from 24 boards taken from three *T. plicata* decks. Using macro- and microscopic characteristics, as well as molecular DNA data, 12 fungal taxa were isolated more than three times. Six species isolated on 1% BMEA were identified as basidiomycetes. One zygomycete and five ascomycetes were identified based on their sporangia and asexual structures, respectively (Table 1). The initial fungal identification was complemented by ITS sequence analyses. ITS sequences often diverge at the species level, and are preferentially used for identification (Schmidt and Moreth 2002, 2003; Högberg and Land 2004). Except for one ascomycete species (*T. plicata* F-A1), sequence analyses confirmed morphological identifications, and allowed assignment of fungi that could not be assigned to known species on a morphological basis (Table 1). *T. plicata* F-A1 had no distinct asexual morphology on MEA media and its ITS sequence showed 95% sequence similarity to unknown endophytic fungi and leaf litter ascomycetes. The basidiomycetes most frequently isolated were *Acanthophysium lividocaeruleum* and *Pachnocybe ferruginea*. These fungi were present in both decay areas and sound inner areas, which tend to have higher extractives content, as extractives in the inner regions are less prone to UV photodegradation and subsequent depletion by leaching (Coombs and Trust 1973; Hon 1991; Shibata

Table 1 Fungal isolates from *T. plicata* decks in service and fungal growth inhibition by *T. plicata* feeder strips.

Fungi	ITS accession number	Source (n)				Growth (mm day^{-1})		Inhibition (%)
		A	B	C	Location	Control	FS	
Acanthophysium lividocaeruleum	AY618666	5	3	4	D and S	2.4	0.9	64.4
Coniophora puteana	DQ516523	2	1	–	D	2.6	1.1	56.0
Dacrymyces stillatus	DQ516524	1	2	2	D	0.6	0.1	83.5
Hyphoderma praetermissum	AY618668	3	–	2	D	1.7	0.1	94.5
Pachnocybe ferruginea	AY618669	9	14	7	D and S	0.8	0.8	8.1
Phellinus ferreus	DQ516525	2	–	5	D	2.2	0.1	95.6
Aureobasidium pullulans	DQ516526	2	–	2	D	0.3	0.1	69.4
Exophiala heteromorpha	DQ516527	5	–	5	D	1.4	0.4	71.4
Phialocephala dimorphospora	AY618688	12	8	9	D	2.1	0.3	86.6
Rhinocladiella atrovirens	AY618683	20	10	12	D	0.6	0.2	71.7
T. plicata F-A1	AY618686	22	5	12	D	1.3	0.2	82.1
Umbelopsis autotrophica	DQ516528	2	3	1	D	2.4	0.2	90.3

A, 100-year-old deck; B, 20-year-old deck; C, 25-year-old deck. From each source, approximately 150 isolations were made. Site of isolation: D, decay areas; S, sound inner areas. Average growth rate calculated after 21 days on 1% MEA without or with *T. plicata* feeder strip (FS). Values are the mean of three replicates.

et al. 2003). The ascomycetes *Phialocephala dimorphosphora* and *Rhinocladiella atrovirens* and the species *T. plicata* F-A1 were commonly isolated, but were present mainly in decay areas. It is likely that these species occupy decay areas because they have low or moderate extractive resistance, since decay pockets contained lower extractive concentrations compared to sound areas. The four known fungal species reported here have also been isolated from *T. plicata* products in service (Scheffer et al. 1984; Wang and Zabel 1990).

One or two representatives from each of the 12 taxa were used to determine tolerance to *T. plicata* extractives. When grown on 1% MEA with *T. plicata* FS, most isolates showed less growth than the controls (Figure 1). Several of the extractives are water-soluble and diffuse from the *T. plicata* FS into the media when placed in contact. In addition, *T. plicata* FS were manufactured in such a way that the transverse wood face was in contact with the media. This cut promotes an intense infusion of moisture into the vesicles and tracheids of the wood and facilitates the diffusion of extracts into the media. *P. ferruginea* exhibited the highest tolerance to *T. plicata* FS extractives; its growth rate was comparable to that of controls. This species was the most common in this work, in agreement with the findings of Wang and Zabel (1990) and Lim et al. (2005), who reported that this species was frequently found on *T. plicata* fences and creosote-treated *T. plicata* poles. It was also found in the heartwood of Douglas fir and was characterized by Kropp and Corden (1986). Two basidiomycetes, *A. lividocaeruleum* and *Coniophora puteana*, and three ascomycetes, *Aureobasidium pullulans*, *Exophiala heteromorpha*, and *R. atrovirens*, showed growth of 28–44% relative to controls, which represents moderate tolerance to *T. plicata* extractives (Table 1). Although *C. puteana* had higher *T. plicata* extractive tolerance than *A. lividocaeruleum*, it was found in or near decay pockets and was less frequently isolated. *A. lividocaeruleum*, one of the species most frequently isolated from *T. plicata* fences, was suggested as a pioneer species with *P. ferruginea* based on their tolerance to β-thujaplicin (Lim et al. 2005). *A. pullulans, E. heteromorpha* and *R. atrovirens* were detected near or in decay pockets. *A. pullulans* can colonize the weathered surfaces of wood from a variety of tree species, including *T. plicata*, and causes black staining (Schoeman and Dickinson 1997; Chedgy et al. 2005). It may play an important role in colonizing and modifying wood surfaces rather than inner areas of *T. plicata* products. The growth of other fungi was significantly reduced on plates with *T. plicata* FS. The only zygomycete, *Umbelopsis autotrophica*, showed very low tolerance to extractives and thus was unlikely to have the ability to modify extractives and so to cause decay of wood.

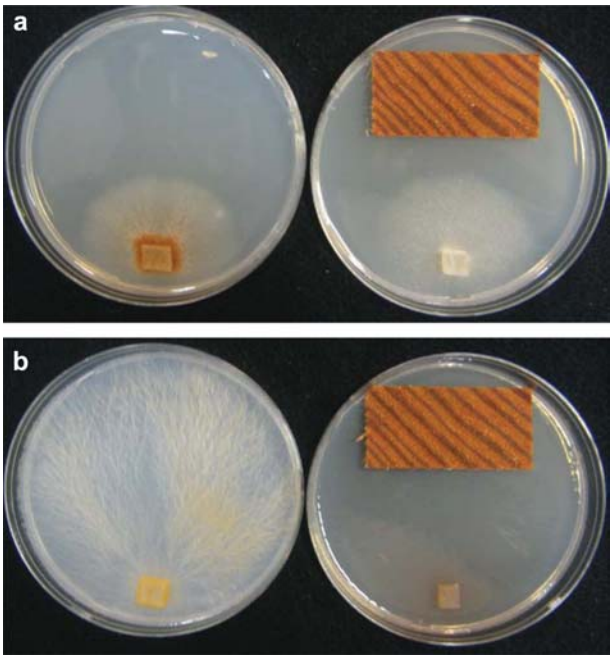

Figure 1 (a) *Pachnocybe ferruginea* and (b) *Phellinus ferreus* growth on 1% malt extract agar (control) and on 1% malt extract agar with *T. plicata* feeder strips after 21-day incubation at room temperature.

It has been reported that the pattern of fungal colonization on surfaces of *T. plicata* products consisted of staining fungi, followed by soft-rot and decay fungi (Banerjee and Levy 1971; Clubbe 1980). The inner sound areas of *T. plicata* boards contained no staining or soft-rot fungi, only extractive-tolerant basidiomycetes (e.g., *P. ferruginea*). We suggest that these species may play a pivotal role in the degradation of the inner parts of *T. plicata* products.

Extractives analyses of *T. plicata* FS

Wood is a naturally variable material and its extractive concentrations vary within and between trees. We observed a similar trend in our control *T. plicata* FS. The total extractive content of *T. plicata* FS was approximately 3.3%, and the five compounds quantified in this work (Table 2) represented approximately 67% of this total amount. The overall concentration of the five extractives varied from 16.86 to 19.24 mg g^{-1}, with a variation of 14%. The amount of active extractives available for inhibition was determined after extract analysis of *T. plicata* FS placed on MEA by difference. It was found that 57.5% of the measured extractives diffused into the media. Among the five compounds investigated, γ- and β-thujaplicin leached out faster than plicatic and thujic acids or β-thujaplicinol (Table 2). ANOVA showed that the treatment effect was significant for each of the five extractives. A significant diffusion of extractives took place ($\alpha = 0.05$) from the wood into the media.

The effects of the five pure compounds in focus on fungal growth on MEA were also examined. Each compound was checked at a concentration equivalent to its loss from the FS (Figure 2, Table 2). Fungi with high (*P. ferruginea*), moderate (*R. atrovirens*) and low tolerance (*P. ferreus*) to *T. plicata* extractives were selected (Table 2). *P. ferruginea* showed high tolerance to all five compounds. Growth of *R. atrovirens* was inhibited by γ-thujaplicin, and reduced to a level similar as for FS by β-thujaplicin and thujic acid. *P. ferreus* did not grow on MEA containing γ-thujaplicin, β-thujaplicin or thujic acid, and its growth was slightly inhibited by plicatic acid and β-thujaplicinol. β-Thujaplicinol, a very effective natural fungicide (Barton and MacDonald 1971), slightly affected

Table 2 Concentration of extractives leached into media and their effect on fungal growth.

Treatment	Media conc. (ppm)	Growth rate (mm day^{-1})		
		P. ferruginea	*R. atrovirens*	*P. ferreus*
Control	–	0.9 (0.1)	0.6 (0.0)	2.2 (0.3)
Plicatic acid	149.4	0.9 (0.0)	0.6 (0.0)	1.9 (0.1)*
γ-Thujaplicin	34	0.8 (0.1)	0.0 (0.0)*	0.0 (0.0)*
β-Thujaplicin	27.6	0.7 (0.0)	0.2 (0.0)*	0.0 (0.0)*
β-Thujaplicinol	5.6	0.8 (0.0)	0.5 (0.0)*	1.8 (0.1)*
Thujic acid	107	0.8 (0.0)	0.2 (0.1)*	0.0 (0.0)*
T. plicata FS		0.8 (0.0)	0.2 (0.0)*	0.0 (0.0)*

Malt extract agar (MEA, 1%) was infused with extractives at the concentration that they accumulated in media having leached from *T. plicata* FS. Media were then inoculated with three representative fungal species and the growth rate (mm day^{-1}) was calculated.
*Growth rate significantly different from the control ($\alpha = 0.05$) following ANOVA.

the growth of the three fungi; however, its concentration in MEA resulting from FS leaching was much lower than the concentration previously tested (Rennerfelt 1948; Roff and Whittaker 1959; Lim et al. 2005). Plicatic and thujic acids have been reported as having low antimicrobial activity (Rennerfelt 1948; Barton and MacDonald 1971). Consistent with this, relatively high concentrations (up to 150 ppm) of plicatic acid did not affect fungal growth significantly; however, high concentrations of thujic acid (107 ppm) inhibited growth of the less tolerant basidiomycetes. Many other chromatographic peaks are present in HPLC chromatograms of the FS, and some of these unidentified compounds diffused into the MEA (data not shown). A note of caution should be observed: it is obvious that more compounds than those tested in this work have inhibitory effects (Barton and MacDonald 1971). Several of these remain uncharacterized. The purification of unknown substances is difficult, and even after identification they are not available commercially. Given this, the microbial toxicity of all extractives of *T. plicata* and their synergistic effects could not be assessed in the present study.

Thujaplicins also occur in plant species throughout the Cupressaceae family (Zavarin et al. 1967). Tropolone-type compounds are known to have antimicrobial activities in species such as Aomori hiba cedar (*Thujopsis dolabrata*) (Inamori and Morita 2001), Mexican cypress (*Cupressus lusitanica*) (Zhao et al. 2006) and Taiwan incense cedar (*Calocedrus formosana*) (Ono et al. 1998).

Ascomycetes may appear more tolerant to β-thujaplicin than basidiomycetes based on literature data. Nevertheless, basidiomycete tolerance has been characterized for relatively few species (Morita et al. 2004). The basidiomycete *P. ferruginea* has often been misidentified because its morphology is graphium-like. In this work we show that this species is highly tolerant to *T. plicata* extractives. Currently, there is no information on the detoxifying mechanism of this fungus against *T. plicata* extractives. Only Jin et al. (1988) have reported on fungal detoxification of thujaplicins. These authors suggested that the fungi isolated from *T. plicata* heartwood (*Kirschsteiniella thujina*, a Sporothrix sp., and a *Phialophora* sp.) could convert thujaplicins into a nontoxic lactone termed "thujin". However, many groups

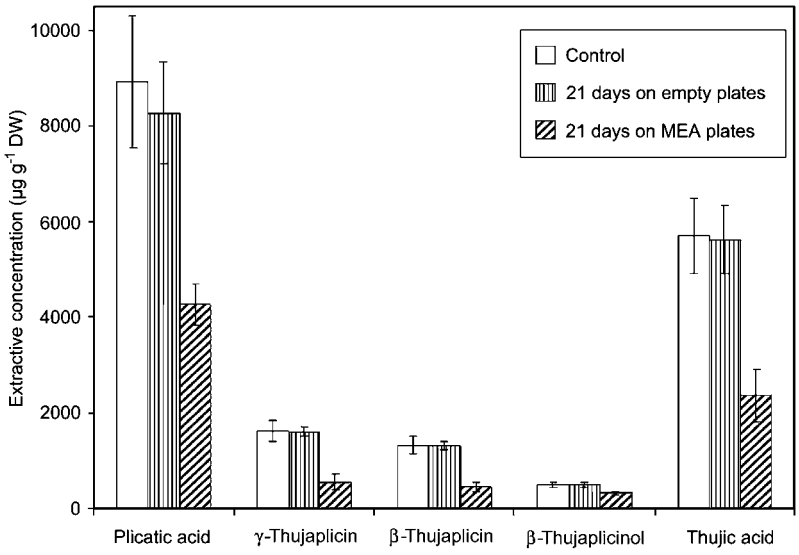

Figure 2 Extractive concentrations (μg g^{-1}) versus *T. plicata* feeder strip (FS) storage conditions. Numbers above bars express the concentration of extractives compared to the control *T. plicata* FS as a percentage. ANOVA data are based on the amount of individual extractive compounds determined by difference (extractive content of *T. plicata* FS before and after exposition to malt agar). Critical F values ($\alpha = 0.05$) were as follows: plicatic acid, $F_{(2,15)} = 35.97$; γ-thujaplicin, $F_{(2,15)} = 79.34$; β-thujaplicin, $F_{(2,15)} = 87.90$; β-thujaplicinol, $F_{(2,15)} = 28.35$; and thujic acid, $F_{(2,15)} = 44.57$. Values are considered significant if the critical F value is greater than the tabular value of $F_{(2,15)} = 3.68$.

have failed to isolate similar fungi from *T. plicata*. The characterization of "pioneer" species such as *P. ferruginea* able to detoxify *T. plicata* extractives will be the topic of further investigations.

Conclusions

Feeder strips prepared from sound *T. plicata* heartwood represent a good tool for screening fungi tolerant against extractives. Results indicate that *P. ferruginea* is an extractive-tolerant pioneer fungus that may play an important role in the initial modification or detoxification of *T. plicata* extractives in wood products. These issues are being addressed in ongoing work.

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