BLACK STAIN OF WESTERN RED CEDAR (*THUJA PLICATA* DONN) BY *AUREOBASIDIUM PULLULANS:* THE ROLE OF WEATHERING

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ABSTRACT

Western red cedar (*Thuja plicata* Donn) is valued for its natural durability conferred by fungicidal extractive chemicals. However, weathered surfaces of *T. plicata* products are susceptible to black stain caused by fungi such as *Aureobasidium pullulans.* The effect of weathering on extractive concentrations at the wood surface was characterized and correlated with the ability of this fungal species to colonize weathered surfaces. UV plus water spray treatments substantially reduced extractives but did not promote fungal colonization. In contrast, UV-only treatments reduced extractive contents less than the other treatments but stimulated fungal colonization. *A. pullulans* exhibited high tolerance to the tropolone β-thujaplicin *in vitro;* thus loss in tropolone content may not be required for colonization. Water spray most likely washed away products of lignin photo-degradation, which resulted in decreased fungal colonization.

Keywords: *Aureobasidium pullulans,* black stain, extractive resistance, weathering, western red cedar (*Thuja plicata*).

INTRODUCTION

Western red cedar (*Thuja plicata* Donn) is utilized in the manufacture of wood products for exterior residential applications. It is valued for its natural durability conferred by fungicidal extractive compounds (Barton and MacDonald 1971). However, *T. plicata* products are susceptible to black stain caused by staining fungi, which can significantly reduce wood aesthetic

qualities and lead to premature replacement. *Aureobasidium pullulans* (de Bary) G. Arnaud causes black stain and is a major colonizer of weathered wood surfaces (Schoeman and Dickinson 1997) and painted wood (Bardage and Bjurman 1998; Jakubowsky et al. 1983; O'Niel 1986 Shirikawa et al. 2002). It can also penetrate many protective coatings (Sharpe and Dickinson 1992b). The black coloration is attributed to the presence of melanin in the fungal hyphae (Yurlova et al. 1999) which protects fungal cells from the damaging effects of UV radiation (Kawamura 1999).

Weathering of the wood surface promotes *A. pullulans* growth since the fungus metabolizes the complex aromatic organic molecules that are formed as a result of lignin photo-degradation (Bourbonnais and Paice 1987; Dickinson 1972; Schoeman and Dickinson 1997; Sharpe and Dickinson 1992a, 1993). This may provide a significant competitive advantage to this fungal species and may explain why *A. pullulans* is predominantly isolated from weathered surfaces (Dickinson 1972). Many of the fungicidal extractive compounds present in *T. plicata* are aromatic and polyphenolic in nature (Barton and MacDonald 1971). Of the array of *T. plicata* extractive compounds characterized to date are a series of compounds known as the tropolones. Several of these compounds, namely the thujaplicins (α-, β-, and γ-thujaplicin) are reported to exhibit strong antimicrobial activity against a range of wood-inhabiting and plant pathogenic fungi (Inamori et al. 2000; Morita et al. 2004).

Extractive contents at the surface of *T. plicata* products may form the first line of defense in preventing fungal spore germination. At this early developmental stage, microorganisms may be less resistant to the toxic effect of extractives. However, extractives near the wood surface may be prone to leaching from precipitation (Chedgy et al. 2005) and are degraded by ultra-violet (UV) radiation from the sun (Coombs and Trust 1973; Shibata et al. 2003) that penetrates the upper 0.75 μm of the wood surface (Hon 1991). It is not known whether *A. pullulans* is tolerant to *T. plicata* extractives or if they are simply depleted by weathering at exposed surfaces, paving the way for colonization. To address *T. plicata* black stain, it is necessary to understand the interactions between *T. plicata* extractives, weathering, and fungal colonization. Therefore, the aims of this research were to 1) establish whether or not *A. pullulans* has resistance to fungicidal tropolone compounds present in *T. plicata in vitro,* and 2) assess the ability of this fungal species to colonize weathered wood surfaces.

METHODOLOGY

Isolation and identification of black stain fungi

Black stain fungi were isolated from in service *T. plicata* siding located in Vancouver, B.C., Canada. Wood flecks that exhibited visible signs of black stain were removed from the surface of siding and placed onto 1% malt extract agar (MEA) plates, then incubated at 20°C for several weeks. Fungi growing on MEA were routinely subcultured from mycelial margins to new MEA plates to obtain pure cultures. Fungal identification was achieved by macro- and micro-morphological analyses using taxonomic guides (de Hoog and Yurlova 1994). This was complemented by molecular technique for species identification as described by Lim et al. (2005). The internal transcribed spacer (ITS) region was used for molecular identification and amplified using the primers ITS5 and ITS4 (Schmidt and Moreth 2002; White et al. 1990). Sequencing from three representative strains of each isolated taxon was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc., CA) at the DNA synthesis and Sequencing Facility, MACROGEN (Seoul, Korea).

β-*Thujaplicin resistance*

Two isolated black stain fungal species, *A. pullulans* and *Hormonema dematioides* Lagerb & Melin, were tested. Mycelial growth was measured on 25-ml MEA plates containing various concentrations of β-thujaplicin (99% pure, Sigma-Aldrich Ltd, ON). A 10 mg ml^{-1} stock solution prepared in 50% ethanol was filter-

sterilized and kept in the dark at 4°C. Concentrations of 0, 2, 8, 16, 32, and 64 ppm (parts per million) were added to MEA. Control MEA plates with and without ethanol showed that ethanol had no effect on fungal growth at the low concentrations used to prepare the β-thujaplicin plates. Media were inoculated with a 5-mm plug of agar taken from the edge of actively growing isolate colonies. The cultures were maintained in the dark at 20°C, and the growth (mm) was evaluated by measuring two perpendicular diameters of the colony after 21 days. This experiment followed a completely randomized design (CRD) with treatments defined as the various β-thujaplicin concentrations ($k = 6$). Three replicate plates were used per strain at each concentration, and three strains of each species were used ($n = 9$). Statistical analysis was performed on data obtained in accordance with this experimental design.

Weathering and fungal effects on wood chemistry

Wood samples.—T. plicata outer heartwood was obtained from a 136-year-old standing tree harvested at the UBC Malcolm Knapp research forest, Maple Ridge, BC, Canada. All wood samples originated from a single longitudinal axis parallel with growth rings. Siding pieces were manufactured of dimensions 160-mm × 65-mm × 10-mm, flatsawn, with the radial face on the largest face. Ponderosa pine (*Pinus ponderosa* P. & C. Lawson) sapwood was also used as a control. The sapwood of this species contains small amounts of extractives that are not fungicidal like those of *T. plicata.*

Weathering of siding material.—T. plicata and *P. ponderosa* siding pieces were exposed to simulated weather conditions using a Weather-Ometer® (Ci65A, Atlas Material Testing Technology LLC, IL) located at the Forintek Canada Corp. laboratory, BC, Canada. Four treatments were used: i) water spray (WS), ii) UV, iii) WS and UV, and iv) no weathering (Table 1). The duration of each weathering regime was 200 h. The WS rate was approximately 9.08 liters per hour. The UV wavelength was set at 340 nm, and we measured 120Kj/m^2 total for each of the 200 h weathering cycles with UV. Wood samples were placed in a spherical drum that rotated around a central UV lamp and water sprinkler system. Samples were arranged at a perpendicular angle to the UV lamp/water source at a distance of approximately 40 cm. Each treatment was run continuously for a period of 200 h, at a chamber temperature of 50°C to prevent mold growth and a relative humidity ranging from 40–95% depending on treatment to provide some moisture to facilitate chemical reactions. It was not possible to measure the wood surface temperature during each weathering regime; instead we were only able to measure the chamber temperature during each regime via an internal thermometer. Eighteen *T. plicata* and *P. ponderosa* siding pieces were subjected to each of the weathering treatments. Each treatment was repeated three times with a new set of samples. Following the weathering process, twelve *T. plicata* and *P. ponderosa* pieces from each treatment were used for chemical analyses.

For chemical analysis, the upper 1 mm of the weathered surface was removed using a computer-controlled Precix 3600 router (Precix, BC). Four pieces were processed each time, and the shavings from these samples were combined. This reduced the number of replicates from

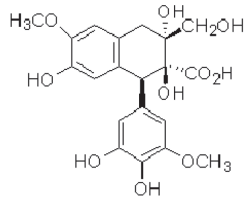
TABLE 1. *Weathering treatments.*

	Program cycle			
	1	2	3	4
Control cycle	—	—	—	—
WS only	30 mins WS (dark)	30 mins (dark)	30 mins WS (dark)	30 mins (dark)
UV only	30 mins UV	30 mins (dark)	30 mins UV	30 mins (dark)
UV and WS	30 mins UV	30 mins WS (dark)	30 mins UV and WS	30 mins (dark)

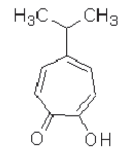
twelve to three for each weathering treatment. Samples were placed into clean glass vials in methanol and extracted for 120 minutes at an ultrasonic frequency of 40 kHz using a Branson 8510 ultrasonic bath (Branson Ultrasonics Corp., CT). A temperature of 4°C was maintained throughout the ultrasonication process by the addition of ice to the water bath, and a lid was used to shield samples from incandescent light to avoid thermal and photo-degradation of extractive compounds of interest. Extract solutions were filtered using a 25-mm 0.2 μm nylon syringe filter to remove any wood particles and stored at 4°C in the dark.

Reverse phase HPLC analysis.—Separation and quantification of extractive compounds were carried out by reverse phase high performance liquid chromatography (HPLC) coupled with an ultraviolet detection system as described in Chedgy et al. (2007) and Daniels and Russell (2007). Five extractives of interest were quantified (μg/g DW) by comparing analyte response with the response factor of an internal standard by a single point calibration. Extractives of interest were (−)-plicatic acid, γ-thujaplicin, β-thujaplicin, β-thujaplicinol, and thujic acid (Fig. 1). A Waters 2695 HPLC separation module (Waters Corp., MA) equipped with an Intersil ODS3 C-18 (3 μm, 4.6 mm × 150 mm) reverse phase separation column (Intersil Corp., CA) was used for extractive separation. Mixed extracts were injected (15 μl) onto the column and separated based on their hydrophobic character using a mobile phase of 0.1% formic acid, 10% acetonitrile, and 89.9% nano-pure H_2O which were run against an increasing linear gradient of 99.9% acetonitrile with 0.1% formic acid. The column chamber was heated to a temperature of 50°C, and a Dionex AD20 UV absorbance detector (Dionex Corp., CA) at wavelength of 230 nm was used for the extractive detection. A total elution of extractives was typically reached after approximately 48 min and MassLynx analytical software (version 4.0, Micromass Ltd, UK) was utilized for chromatographic analysis.

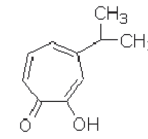
A CRD was used to assess the effect of weathering on extractive concentration. Experimental treatments were defined as the different weathering treatments ($k = 4$). The surface wood was analyzed from twelve replicate siding pieces from each weathering treatment. Surface wood was removed from four pieces simultaneously using the automated router and the dust was



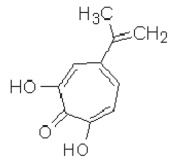
2,3,6-Trihydroxy-7-methoxy2hydroxy methyl-4-(3',4'-dihydroxy-5'-methoxy phenyl)-tetralin-3-carboxylic acid
(-)-Plicatic acid



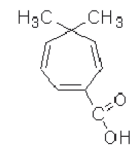
2-Hydroxy-5-isopropyl-2,4,6-cycloheptatrien-1-one
γ-Thujaplicin



2-Hydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one
β-Thujaplicin



2,7-Dihydroxy-4-isopropyl-2,4,6-cycloheptatrein-1-one
β-Thujaplicinol



7,7-Dimethylcycloheptatriene-3-carboxylic acid
Thujic acid

FIG. 1. Chemical structure and nomenclature used for *T. plicata* heartwood extractive compounds of interest.

pooled together. This reduced the replicate number from twelve to three. Therefore, $n = 9$ (3 × analytical surface wood samples, 3 × weathering treatments).

Inoculation of A. pullulans on weathered sidings

Weathered and un-weathered (control) *T. plicata* and *P. ponderosa* siding pieces were sprayed with a liquid culture of *A. pullulans* and incubated in growth chambers. Three *A. pullulans* strains were grown in 250-ml flasks containing 100 ml of 1% malt extract media. The inoculum was approximately 1×10^6 spores. This was prepared by slicing 10- × 1-cm^2 squares from 1% MEA pure culture plates and submersing them in 5 ml of distilled H_2O in a sterilized 15-ml falcon tube and vortexed. Spores were separated from mycelium by pouring the mixture through sterilized cheesecloth. The filtrate was subjected to two successive rounds of centrifugation at 6000 g for 5 min and the pellet re-suspended in 5 ml of distilled H_2O to ensure adequate washing of spores. Spore density was estimated using a hematocytometer from La Fontaine (La Fontaine, Germany) in accordance with the manufacturer's guidelines.

Shake cultures were incubated at 22°C, 200 rpm, for fourteen days. Cells were collected by centrifugation and re-suspended in nano-pure H_2O. The culture suspension from three *A. pullulans* strains were mixed together in equal parts. The resulting solution was sprayed homogeneously over all of the weathered siding pieces. Six *T. plicata* and *P. ponderosa* siding pieces from each weathering treatment were completely randomized and divided among four growth chambers. Samples were suspended over a layer of water. The incubation was carried out for 6 months at 20°C and close to 100% relative humidity. To provide a surface film of water, the samples were sprayed with a distilled water mist every 3–4 days. The degree of colonization was assessed in two ways: 1) qualitatively, by scoring siding pieces to reflect the degree of black staining; 2) quantitatively, using a Minolta CM-2600d portable integrated sphere spectrophotometer (Konica-Minolta Ltd, ON) which yields an index of white-black coloration ranging from 0–100 (0 = Black, 100 = White). Discoloration of wood after exposure to *A. pullulans* could be calculated by taking readings before and after colonization at six regions of each siding piece. As before, a CRD was used to assess the effect of weathering on the colonization ability of *A. pullulans* on weathered surfaces. Experimental treatments were defined as the different weathering treatments ($k = 4$). Six siding pieces were analyzed for the degree of discoloration from each weathering run, and each weathering treatment was repeated three times ($n = 18$) (6 replicate siding pieces × 3 weathering treatments).

Statistical analysis

All experiments followed a completely randomized design (CRD) with treatments denoted as k and replicates as n. Analysis of variance (ANOVA) ($\alpha = 0.05$) and Tukey's test for comparison of means (Tukey 1949) were performed with this experimental design. Treatment effects were considered significant at the 95% significance level if the resulting critical F value was greater than the appropriate tabular value ($F_{[(k-1)],[k(n-1)]}$). All statistical analyses were performed using JMP IN software (version 4.0.3 (academic), SAS Institute Inc., NC).

RESULTS AND DISCUSSION

Macro- and microscopic characterization of the fungal isolates, along with the ITS sequences allowed us to recognize two fungal taxa, *Aureobasidium pullulans* and *Hormonema dematioides*. *Aureobasidium* and *Hormonema* species are related to the bitunicate Ascomycete family Dothideaceae and form black, yeast-like cells (Yurlova et al. 1999). Because both species have similar morphological characteristics and cannot be easily differentiated (Takeo and de Hoog, 1991), we sequenced ITS regions as suggested by Ray et al. (2004) to confirm their identification. Sequence dissimilarity was not observed within the species, but was found between the two species at a level of 12.38%. *A. pullulans* was the most commonly isolated species and our results agreed with previous research (Dickinson

1972; Bardage and Bjurman 1998), but only a few strains (three) of *H. dematioides* were isolated from *T. plicata* siding. The ITS sequences of our isolates have been deposited in GenBank with the accession numbers DQ787427 (*A. pullulans*) and DQ787428 (*H. dematioides*).

Resistance to β-hujaplicin *in vitro* was assessed for both *A. pullulans* and *H. dematioides.* Statistical analysis suggested that β-thujaplicin concentration had a significant effect on the growth of both *A. pullulans* and *H. dematioides* (Table 2). Tabular numbers in parentheses indicate standard deviation (mm). ANOVA indicated that treatment effect was significant for both *A. pullulans* ($F_{(5,48)} = 324.59$) and for *H. dematioides* ($F_{(5,48)} = 559.55$). Values were considered significant if greater than the tabular value of $F_{(5,48)} = 2.41$ ($\alpha = 0.05$). Numbers followed by the same letter were not significantly different ($\alpha = 0.05$) according to Tukey's test of multiple comparison of means.

A. pullulans isolates exhibited high tolerance to β-thujaplicin. They were able to grow at 32 ppm (5.5 ± 1.1 mm), although growing more slowly than the controls (33.3 ± 1.5 mm). Similar tolerance was observed with some pioneer and decay fungi identified in *T. plicata* standing trees and other wood products (Lim et al. 2005, 2007). *H. dematioides* showed little β-thujaplicin resistance, with growth completely inhibited at 8 ppm and severely impaired at 2 ppm (14.7 ± 2.1 mm) compared to the control (31.9 ± 1.8 mm).

The five extractives of interest were quantified following analysis of the upper 1 mm of *T. plicata* wood surfaces following different weathering treatments (Fig. 2). Y error bars indicate standard deviation. Numbers above error bars represent concentration expressed as a percentage of the control. Critical F values are as follows for each extractive of interest: plicatic acid ($F_{(3,32)} = 68.24$); γ-thujaplicin ($F_{(3,32)} = 253.47$); β-thujaplicin ($F_{(3,32)} = 615.80$); β-thujaplicinol ($F_{(3,32)} = 292.25$); and thujic acid ($F_{(3,32)} = 397.81$). Values were considered significant if greater than the tabular value of $F_{(3,32)} = 2.90$ ($\alpha = 0.05$). Weathering was found to have a significant effect on extractive concentration. Overall losses for the five extractives measured were 29.8%, 79.9%, and 89.4% for UV, WS, and UV + WS treatments, respectively. UV alone had the least effect on extractive content.

Our data were consistent with the results of Johnson and Cserjesi (1980) for the depletion of β- and γ-thujaplicin in *T. plicata* shakes exposed in natural conditions in Vancouver, BC, Canada. They established that β- and γ-thujaplicin depletion was 25% after a year of exposure and 90% after three years, although this may have been due in part to biodegradation. Extractive loss in our UV + WS treatment using a weather-Ometer was approximately equivalent to one year's weathering loss in natural conditions. Johnson and Cserjesi (1980) also noted that the β- to γ-thujaplicin concentration ratio (average 1:1.5) remained constant during weathering. Compounds such as β-thujaplicin are prone to photodegradation. Shibata et al. (2003) demonstrated that irradiation (210-380 nm) caused photochemical decomposition of β-thujaplicin and a measurable loss of antibacterial activity.

TABLE 2. *Total fungal growth (mm) of black staining isolates after 21 days on MEA containing various concentrations of* β*-thujaplicin.*

	β-Thujaplicin concentration (ppm)					
	0	2	8	16	32	64
A. pullulans 1	33.33 (1.53)[a]	31.00 (0.93)[a]	26.67 (2.31)[b]	9.33 (2.08)[c]	5.00 (1.00)[d]	—
A. pullulans 2	32.33 (2.52)[a]	32.33 (2.67)[a]	22.67 (5.03)[b]	11.50 (0.5)[c]	5.17 (1.04)[d]	—
A. pullulans 3	34.33 (0.58)[a]	30.33 (2.47)[a]	25.33 (2.52)[b]	10.67 (0.58)[c]	6.33 (1.15)[d]	—
Mean	33.33 (1.54)[a]	31.22 (2.03)[a]	24.89 (3.29)[b]	10.50 (1.05)[c]	5.50 (1.07)[d]	—
H. dematioides 1	30.00 (2.00)[a]	15.00 (3.25)[b]	—	—	—	—
H. dematioides 2	32.33 (0.58)[a]	14.67 (2.09)[b]	—	—	—	—
H. dematioides 3	33.33 (2.87)[a]	14.33 (0.9)[b]	—	—	—	—
Mean	31.89 (1.82)[a]	14.66 (2.08)[b]	—	—	—	—

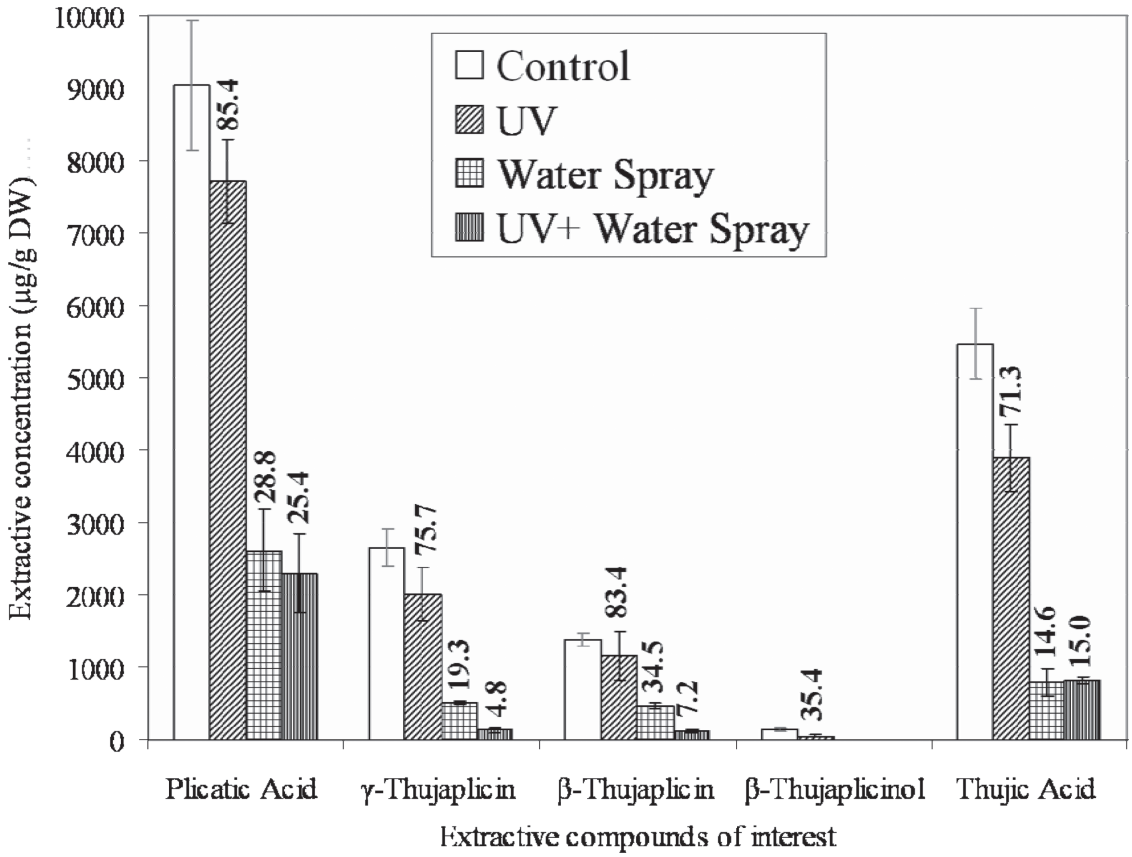


FIG. 2. Weathering treatment effects on the concentrations (µg/g DW) of *T. plicata* extractive compounds of interest.

We assessed whether *A. pullulans* was likely to colonize *T. plicata* when extractives were depleted by weathering compared to un-weathered wood. Growth of *A. pullulans* isolates on weathered siding pieces after six months' incubation was estimated qualitatively and quantitatively. Table 3a shows the mean qualitative growth scores that were assigned by visual inspection following different weathering treatments; 0 = no visible growth; 1 = trace growth, some mild grey/black coloration; 2 = obvious coloration; 3 = significant black stain; 4 = some regions completely covered; 5 = total coverage, no unstained wood. In addition we recorded photograph images of *A. pullulans* growing on weathered surfaces using a stereo microscope (Fig. 3). Growth of *A. pullulans* was greatest on surfaces weathered by UV.

TABLE 3a. *A pullulans growth on weathered wood surfaces estimated qualitatively.*

	Weathering treatment			
	No weathering	UV	UV + WS	WS
P. ponderosa	3.58	4.15	2.21	2.01
T. plicata	2.25	3.71	1.51	1.47

Quantitative assessment of growth using spectrophotometer readings to calculate the mean degree of discoloration of weathered wood before and after colonization by *A. pullulans* agreed with these observations (Table 3b). Tabular numbers in parentheses indicate standard deviation from the three replications of the experiment. Critical F values were as follows: on pine ($F_{(3,68)} = 194.44$) and on cedar ($F_{(3,68)} = 25.72$). These values were much greater than the tabular value of $F_{(3,68)} = 2.74$ ($\alpha = 0.05$). Numbers

TABLE 3b. *Spectrophotometer measurements of weathered wood discoloration before and after colonization by A. pullulans.*

	Weathering treatments			
	Control	UV	UV + WS	WS
P. ponderosa	35.66 (4.47)[a]	31.94 (5.92)[a]	5.15 (2.69)[c]	21.17 (0.88)[b]
T. plicata	8.49 (3.37)[b]	18.98 (7.92)[a]	8.68 (3.37)[b]	8.77 (3.49)[b]

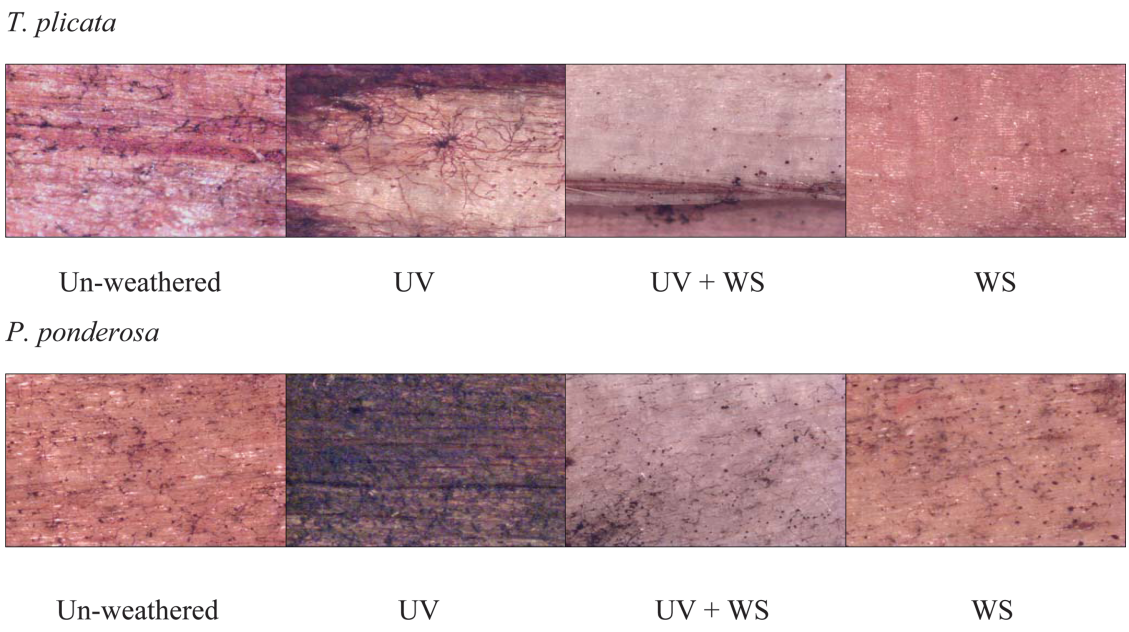


FIG. 3. *A. pullulans* colonization of weathered wood surfaces.

followed by the same letter in each row are not significantly different ($\alpha = 0.05$) according to Tukey's test for multiple comparison of means.

Statistical analysis suggested that treatment effects had a significant impact on the discoloration of weathered surfaces by *A. pullulans* on pine (*P. ponderosa*) and on cedar (*T. plicata*). In every case, *A. pullulans* was successfully re-isolated and identified from all siding pieces used in the trial. As expected, *A. pullulans* grew more vigorously on pine siding than on cedar. Weathering by UV-only gave the greatest degree of fungal colonization and discoloration on both cedar and pine, while treatment by UV + WS gave the least. UV consistently promoted fungal colonization. Lignin is highly susceptible to photo-degradation (Chang 2002; Crestini and Auria 1996). *A. pullulans* can metabolize products of lignin photo-degradation (Bourbonnais and Paice 1987; Schoeman and Dickinson 1997; Sharpe and Dickinson 1992a, 1993). Our data suggested that UV-only caused less decrease in extractive concentrations in contrast to other weathering treatments. *A. pullulans* showed a high tolerance to the fungicidal effects of β-thujaplicin; this suggests that extractive depletion is not necessary for fungal colonization. *A. pullulans* could also colonize un-weathered wood surfaces. Furthermore, the depletion of extractives was greater when water spray was introduced into the treatments and resulted in less colonization than on un-weathered wood surfaces.

CONCLUSIONS

Weathering treatments of *T. plicata* siding caused significant changes in the extractive con-

tent of exposed surface. UV plus water spray severely reduced extractives but did not lead to increased fungal colonization compared to unweathered wood. Water spray most likely washed away products of lignin photodegradation, leaving the wood surface void of accessible carbon sources resulting in decreased fungal growth. *A. pullulans* exhibited high tolerance to the tropolone β-thujaplicin *in vitro,* suggesting that tropolone reductions by weathering may not be required for colonization. It is likely that *A. pullulans* may have competitive advantages in colonizing exposed *T. plicata* surfaces because it can use lignin breakdown products as a carbon source, it is resistant to UV due to its melanized cells and it tolerates tropolones.

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