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Short communication

Inhibition of basidiospore germination by western redcedar heartwood extractives

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ABSTRACT

Understanding the relationship between extractives and decay resistance in western red cedar (WRC) is essential for breeding durable planting stock. To date, most such work has focused on resistance to mycelial attack. However, this is potentially misleading since WRC is largely used in above-ground applications where basidiospore germination is the primary way in which the wood is initially colonized. Efficacy against basidiospores is likely a critical factor affecting wood's decay resistance in above-ground applications. In this study the effect of selected extractives on basidiospore germination was evaluated. Partially-extracted WRC veneer samples, and spruce samples treated with beta-thujaplicin, thujic acid, or plicatic acid were used. Beta-thujaplicin and thujic acid were associated with significantly reduced rates of *Gloeophyllum sepiarium*, *Fomitopsis palustris,* and *Dichomitus squalens* basidiospore germination. Plicatic acid was not associated with any effect on basidiospore germination. Planting stock that generates heartwood with high concentrations of thujaplicins and thujic acid should be selected to yield wood that will be resistant to basidiospore germination.

1. Introduction

Untreated western redcedar (*Thuja plicata* Donn) is used largely in above-ground, exterior applications, such as decking, fencing, and siding. These applications rely on the natural decay resistance of western redcedar (WRC) to meet service life expectations (Cartwright, 1941; Scheffer, 1957; Freitag and Morrell, 2001; Morris et al., 2011; Morris and Ingram, 2013). To date, most research investigating the relationship between extractives and decay resistance has focused on resistance to mycelial attack (Nault, 1988; DeBell et al., 1997; Taylor et al., 2006; Morris and Stirling, 2012; Kirker et al., 2013). This is potentially misleading since basidiospore germination is the primary way in which wood is initially attacked above ground (Savory and Carey, 1976; Schmidt and French, 1979; Bjurman, 1984; Fougerousse, 1984; Hegarty and Buchwald, 1988; Croan, 1994, 1995). Efficacy against basidiospores is likely a critical factor affecting the species' decay resistance. WRC heartwood has been shown to inhibit germination and kill *Gloeophyllum trabeum* (Pers.) Murrill (reported as *Lenzites trabea* (Pers.) Fr.) basidiospores (Morton and French, 1966). It was also reported to be more resistant to colonization by basidiospores of *Phellinus weirii* (Murrill) Gilb. than other species (Nelson, 1976). However, the extractives responsible for this inhibitory and sporicidal activity had not been investigated.

Twenty-one heartwood extractive compounds have been isolated from WRC heartwood and identified. The most abundant extractives are the thujaplicins, plicatic acid, and thujic acid (Daniels and Russell, 2007). The thujaplicins are well-known fungicides with demonstrated activity against mycelial attack (Rennerfelt, 1948; Rudman, 1962, 1963; Morita et al., 2004; Yen et al., 2008; Li et al., 2012). Plicatic acid is reported to have fungistatic activity (Roff and Atkinson, 1954), but has not shown fungicidal efficacy against mycelia at concentrations that would be present in wood (Lim et al., 2007; Stirling et al., 2007; Stirling and Morris, 2016). Thujic acid is generally believed to have low activity against decay fungi (Erdtman and Gripenberg, 1949), but has been reported to inhibit the growth of *Rhinocladiella atrovirens* Nannf. and *Phellinus ferreus* (Pers.) Bourdot & Galzin mycelia growing on artificial media (Lim et al., 2007).

Established mycelia are generally more tolerant to chemicals than basidiospores (Morton and French, 1966; Choi et al., 2002; Woo and Morris, 2010), though exceptions have been noted (Savory and Carey, 1976). Chromated copper arsenate and sodium pentachlorophenate are reported to be effective against

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basidiospores at 10 to 100X lower concentrations than they are against established mycelia of *Coriolopsis gallica* (Fr.) Ryvarden (reported as *Trametes hispida* Bagl.), *Rhodonia placenta* (Fr.) Niemelä, K.H. Larss. & Schigel (reported as *Poria placenta* (Fr.) Cke., and *Gloeophyllum trabeum* (Pers.) Murrill (Schmidt and French, 1979). Assessment of the efficacy of copper against basidiospores showed that fungi whose mycelia were copper-tolerant produced basidiospores that were susceptible to copper (Choi et al., 2002; Woo and Morris, 2010). Aliphatic organic acids have also been shown to prevent germination and kill basidiospores (Schmidt, 1985).

It was hypothesized that one or more WRC heartwood extractive compounds inhibit germination of basidiospores from wood decay fungi. Only beta-thujaplicin, plicatic acid, and thujic acid were available in sufficient quantities for testing at this stage. Understanding which extractives contribute to decay resistance is necessary for selecting planting stock that will yield heartwood that is durable when used in above-ground applications.

2. Materials and methods

2.1. Inoculum preparation

Cultures of *Gloeophyllum sepiarium* (Wulfen) P. Karst., *Fomitopsis palustris* (Berk. & M.A. Curtis) Gilb. & Ryvarden, and *Dichomitus squalens* (P. Karst.) D.A. Reid were obtained from FPInnovations' culture collection. Sporulating cultures were grown using the methods similar to those described by Choi et al. (2001). Some modifications to these methods have been made to encourage sporulation of *G. sepiarium* and *F. palustris* cultures. Two different zones on the growing surface of the plates were created to perturb the growth of fungi across the plate surface. Plates contained an area of malt agar surrounded by water agar, a piece of filter paper placed to one side, and a pine wood plug placed in the water agar, creating nutrient rich and deficient areas. A photograph of the setup for spore production is shown in Fig. 1. Agar plugs were used for inoculation as this allowed sporulation to occur more quickly than with wood inoculum. In addition, for *G. sepiarium*, pine sapwood sawdust, fertilizer (NPK 20/20/20), and oat bran were added to the nutrient media. Plates were incubated for 2 weeks at 24 °C to allow mycelia to grow. The plates were then inverted and placed in a 15 °C and 90% relative humidity incubator for six weeks with cycles of 12 h darkness and 12 h artificial light (fluorescent and UV).

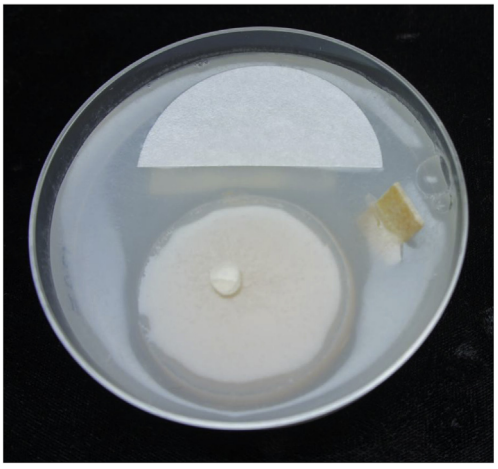

Fig. 1. Photograph of set up for basidiospore production. Each plate contains an area of malt agar surrounded by water agar, a piece of filter paper placed to one side, and a pine wood plug placed in the water agar.

To collect spores for inoculations, sporulating cultures were removed from the growth chamber, a fresh sterile lid was put in place, then the plate was sealed with Parafilm® to prevent contamination and placed back in the growth chamber for two days to allow collection of fresh spores. Sterilized distilled water was used to wash the spores collected from the lid into an inoculum suspension. A small portion of solution was retained to confirm the presence of spores under a compound microscope at high magnification. The number of spores present was counted with a haemocytometer and the spore solution was diluted with sterile distilled water to a 250,000 spores mL^{-1} suspension. A 30 μL spore suspension used for individual inoculation contained approximately 8000 spores. Spore suspension viability was confirmed by growth on malt agar plates.

2.2. Test sample preparation

White spruce (*Picea glauca* (Moench) Voss) heartwood was selected as a substrate to evaluate the impact of selected compounds on basidiospore germination because it is has few extractives, no natural durability, and very low concentrations of nutrients to support fungal growth. White spruce and WRC heartwood were cut into 10 mm × 10 mm × 1 mm samples. White spruce samples were conditioned at 40 °C overnight, then dip-treated in groups of 25 for 5 min at 20 °C with the solutions specified in Table 1. Concentration targets were based on amounts typically found in WRC heartwood (Daniels and Russell, 2007). The target for copper loading was based on concentrations previously reported to control basidiospore germination (Choi et al., 2004; Stirling et al., 2015). The beta-thujaplicin and thujic acid samples were dipped twice to ensure the target uptakes were met. Ethanol was used to prepare beta-thujaplicin and thujic acid treatment solutions. An additional set of samples treated only with ethanol was included as a control in the spore germination tests. Samples were gently agitated with a vortex mixer during dipping to ensure all sample faces were exposed to the treating solution. Western redcedar heartwood samples were Soxhlet-extracted for 6 h with selected solvents as specified in Table 2. To estimate extractives content for the WRC samples, 20 random samples for each treatment were cut up, ethanol-extracted, and analyzed by HPLC (Daniels and Russell, 2007). Total thujaplicins were calculated as the sum of alpha-, beta-, and gamma-thujaplicin.

All samples were conditioned at 40 °C for 24 h, numbered, and randomly sorted into inoculation groups for analysis. Twelve samples from each treatment group were selected for each basidiospore type. A total of 120 samples were evaluated against basidiospores from each test fungus. The samples were double bagged in sealed polyethylene bags and sterilized by 3.4 Mrad of electron beam irradiation.

2.3. Inoculation and inspection

Twenty-four multiwell plates were prepared under sterile conditions with 2 mL of water agar and a polypropylene mesh separator in each well. One sample was aseptically placed in each well and inoculated with 30 μL of basidiospore suspension. Samples were inoculated *in situ* to ensure placement of spore solution on the wood surface. This was repeated for each basidiospore culture. After inoculation the samples were moved to an incubator at 24 °C for two weeks. Each sample was visually inspected for basidiospore germination after 14 days using a dissecting microscope with 10 to 20× magnification. After 21 days, samples without mycelial growth were removed from the well plates and individually inspected under higher magnifications (40× magnification) to ensure