ANTIFUNGAL EFFECTS OF HINOKITIOL AND ITS SODIUM SALT FOR WOOD PROTECTION

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The ability of natural and synthetic hinokitiol, as well as a water soluble derivative (hinokitiol sodium salt), to protect wood against fungal attack was examined. Synthetic and natural hinokitiol provided similar protection. All three materials exhibited similar antifungal activity against *Aspergillus niger* and *Penicillium citrinum* on yellow poplar wafers at concentrations of 1 mg/mL or greater. Fungal attack by *Gloeophyllum trabeum* or *Trametes versicolor* was completely inhibited in soil block tests in wood treated with any of the three extracts at concentrations of 20 mg/mL or greater. The water soluble hinokitiol sodium salt was highly susceptible to leaching, and blocks subjected to leaching had little resistance to fungal attack. The results suggest that further formulation development will be necessary to produce a water-soluble hinokitiol system that can resist leaching and retain biological activity.

Keywords: Hinokitiol; Hinokitiol sodium salt; Wood protection

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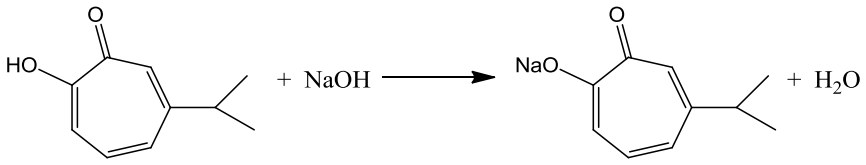
INTRODUCTION

Wood is amongst the most widely used renewable materials, but it performs poorly when it is exposed to fungal and insect attacks. Wood can be supplementally protected with preservatives, but there is a general public concern over the risks of using these compounds. One alternative wood protection approach is the use of natural plant-derived extracts. While some of these compounds have potent activity against a range of both target and non-target organisms, they are generally more acceptable to the general public. Hinokitiol (β-thujaplicin) has long been known to impart resistance to biological attacks and is considered to be the primary compound responsible for the natural durability of Cupressaceae wood (Baya *et al*. 2001; Chedgy *et al.* 2007, Inamuri *et al*. 2000; Ohira *et al*. 1994, 1996; Sakai *et al*. 1997; Yen *et al*. 2008; Yamaguchi *et al*. 1997, 1999; Lim *et al.* 2005). While hinokitiol has exceptional properties, it also has several drawbacks. First, it must be extracted from heartwood residues, and this leads to difficulties in obtaining sufficient quantities of material for commercial use. It is, however, possible to synthesize hinokitiol, eliminating the need to obtain materials for extraction. In addition, hinokitiol is not water soluble. While the use of organic solvents for delivering protectants into wood is not uncommon, such use adds considerable cost to the treatment solution. Fortunately, hinokitiol is an acid that can be saponified by reactions with a base such as sodium hydroxide to form a water-soluble salt (See Eq.1). Some of these compounds are already used in cosmetics, pesticides, or other fields

(Marrone *et al*. 2009; Otsu *et al*. 1997) and may also be useful for wood protection as a water-based protectant.



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In this study, natural and synthetic hinokitiol (β-thujaplicin) as well as Hiba oil (the essential oil from which natural hinokitiol was extracted) and a water soluble derivative (hinokitiol sodium salt), were investigated for their activity against selected mold and decay fungi in laboratory tests. While water solubility can reduce solvent costs during application, it is important to determine if the modified compound retains its efficacy and if the change in water solubility markedly increases susceptibility to leaching once the wood is placed in service.

EXPERIMENTAL

Extract Sources

Synthetic and natural hinokitiol, as well as the hinokitiol salt, were purchased from Osaka Chemicals (Osaka, Japan) (Table 1). In addition, Hiba oil, a mixture of 2 to 3% hinokitiol and β-dolablin, was evaluated. Hiba oil was diluted with ethanol, hinokitiol solutions were diluted with 1:1 ethanol-water (v/v), and the sodium salt solution was diluted with water.

Table 1. Characteristics of Natural Product Extracts Examined for Activity Against Wood Inhabiting Fungi.

Product	Appearance	Physical Properties	Active Component Content
Hiba oil	Yellow oil	b.p. ~256 to 295 °C	~2 to 3% (Hinokitiol + β-dolablin)
Natural hinokitiol	Light yellow crystal	m.p. ~50 to 52 °C	99.9 % Hinokitiol
Synthetic hinokitiol	White crystal	m.p. ~50 to 52 °C	99.9 % Hinokitiol
Na-salt of hinokitiol (H-Na)	Yellow crystal	m.p. ~218.0 to 223.6 °C	99.9 % Na-salt of hinokitiol

Mold Resistance

Yellow poplar (*Liriodendron tulipifera* L.) sapwood wafers (3 mm by 10 by 10 mm long) were cut from sliced veneer. The wafers were conditioned to a constant weight at 20 °C and 65% RH, and then dipped for 60 seconds in the appropriate solution. Excess solution was allowed to drain from the wafers, which were then placed on a plastic screen over 2 layers of moistened filter paper in a glass Petri dish (Li *et al*. 2008). Each treatment was replicated on 10 wafers. The wood surfaces were sprayed until excess inoculum solution ran off the wood surface with a spore/hyphal suspension of *Aspergillus niger* and *Penicillium citrinum* prepared by inoculating separate flasks of 1 percent malt

extract with an agar disk cut from the actively growing edge of cultures of a given fungus. The flasks were incubated at room temperature for 7 to 14 days, and then the mycelium was collected by filtration through a sterile filter. The mycelium was washed with distilled water and then re-suspended in sterile distilled water. The mixtures were combined and briefly blended to macerate the mycelium before it was placed in a sterile spray bottle for application to the wood.

The plates containing the wafers were sealed with a wax film to retard moisture loss and incubated at 28 °C for 2 weeks. The degree of discoloration on each wafer was visually assessed on a scale from 0 (no damage) to 100 (complete discoloration) on the basis of total area covered by fungi.

Initially, *Alternaria alternata, Trichoderma koningiopsis, Paecilomyces lilacinus,* and *Fusarium oxysporum* were also tested. However, there was little evidence of growth by these fungi after 2 weeks of incubation, possibly because the veneer moisture contents were not suitable for these fungi. Only the *A. niger* and *P. citrinum* data will be reported.

Decay Resistance

Cubes (10 mm by 10 mm by 10 mm) of *Populus ussuriensis* Kom. were cut, oven dried at 50 °C, and weighed. The lower temperature was employed to reduce the risk of volatilization of hinokitiol in subsequent drying steps. The extracts were diluted in the appropriate solvent to produce concentrations of 5, 10, 20, and 40 mg/mL of natural or synthetic hinokitiol or the sodium salt solution. Twenty four test blocks to be treated with a given formulation were placed into a one liter basket which was placed in a desiccator. The desiccator was then subjected to a 30 minute vacuum at 0.1 MPa, and then the treating solution was added. Additional blocks were treated with water or ethanol to serve as solvent controls. The remaining vacuum was released and the blocks were then soaked in the treating solution for an additional 8 hours. The blocks were removed from the solution, wiped clean of excess solution, and weighed to determine the net solution absorption. The blocks were then air-dried for 1 week before being oven dried at 50 °C and weighed.

Half of the blocks from each treatment group were subjected to a leaching exposure, as described in the American Wood Protection Standard E10, where the blocks were immersed in distilled water which was subjected to a vacuum (AWPA, 2004). The water was then changed after 6, 24, and 48 hours and then every 24 hours for 2 weeks. The leached blocks were oven-dried and weighed. The leached and non-leached blocks were then heated at 100 °C for 15 minutes to eliminate any microorganisms. The lower sterilization temperature was employed to reduce the risk of heat-damage to the extracts.

The decay resistance of leached and non-leached blocks was evaluated in an AWPA E10 soil block test (AWPA 2004). Glass jars were half-full with moist forest loam, and a poplar feeder strip was placed on the soil surface. The jars were loosely capped and sterilized by heating them at 121 °C for 45 minutes. After cooling, a 3 mm diameter disc of agar cut from the actively growing edge of the test fungus was placed on the edge of the feeder strip. The fungi tested were *Gloeophyllum trabeum* and *Trametes versicolor*, which cause brown and white rot decay, respectively. The bottles were then incubated at room temperature until the feeder was covered by the fungus. The sterilized test blocks were then placed on the feeder strip; the jars were loosely capped and incubated for 12 weeks at 28 °C.

At the end of the incubation period, the blocks were removed, scraped clean of mycelium and soil, and weighed. The difference in weight between the oven-dry weight

and the final weight was used to determine the final moisture content. The blocks were then oven-dried (50 °C) and weighed to determine the wood weight loss over the exposure period, which served as a measure of chemical efficacy.

RESULTS AND DISCUSSION

Mold Resistance

Fungal attack on the untreated controls was rapid, even after only 2 weeks of incubation (Table 2). Among the four hinokitiol-related samples, Hiba oil had the least activity. All of the hinokitiol samples and the sodium salt provided good efficacy against the mold fungi evaluated, at levels above 0.1 mg/mL. Colonization by *A. niger* or *P. citrinum* decreased at concentrations of 0.2 mg/mL or higher. Fungal growth was completely inhibited by hinokitiol and its sodium salt at concentrations of 1.0 or 2.0 mg/mL.

Table 2. Effect of Treatment of Yellow Poplar Veneers with Natural or Synthetic Hinokitiol or Its Sodium Salt on Resistance to *A. niger* or *P. citrinum.*

Sample	Conc. mg/mL	Degree of Discoloration			
		A. niger		*P. citrinum*	
		Area	*Intensity*	*Area*	*Intensity*
Hiba oil	0.1	10(0)	6(2)	10(0)	7(1)
	0.2	10(0)	6(2)	10(0)	6(2)
	0.3	10(0)	6(2)	10(0)	6(2)
	0.5	9(1)	3(2)	10(0)	6(2)
	1.0	3(4)	2(2)	10(0)	5(1)
	2.0	4(4)	2(2)	10(0)	5(2)
Natural Hinokitiol	0.1	10(0)	7(1)	10(0)	5(1)
	0.2	10(1)	7(1)	6(5)	3(3)
	0.3	6(4)	4(2)	5(3)	4(2)
	0.5	4(3)	4(2)	5(2)	4(2)
	1.0	0(0)	0(0)	0(0)	0(0)
	2.0	0(0)	0(0)	0(0)	0(0)
Synthetic Hinokitiol	0.1	10(0)	6(1)	10(0)	5(2)
	0.2	10(0)	6(1)	8(3)	3(2)
	0.3	5(4)	4(3)	5(4)	2(2)
	0.5	4(3)	3(2)	5(2)	3(2)
	1.0	0(0)	0(0)	0(0)	0(0)
	2.0	0(0)	0(0)	0(0)	0(0)
Hinokitiol Na	0.1	10(0)	7(1)	10(0)	6(2)
	0.2	10(0)	7(1)	8(4)	4(2)
	0.3	10(0)	6(2)	6(5)	3(2)
	0.5	6(3)	5(3)	5(3)	4(2)
	1.0	0(0)	0(0)	0(0)	0(0)
	2.0	0(0)	0(0)	0(0)	0(0)
CK	0.0	10(0)	5(2)	10(0)	6(2)

Values represent means of ten replicates per treatment while numbers in the parentheses are one standard deviation. Value can range from 0 (no fungal growth) to 10 (completely covered or very high intensity of damage).

There were no differences in performance between synthetic or natural hinokitiol. The poor performance of Hiba oil led us to drop this from further testing, but hinokitiol clearly has potential for limiting mold attack of wood.

Decay Tests

Net weight gains in blocks generally increased with an increasing of the solution concentration, and there was little difference in the solution uptake between the synthetic and natural hinokitiol (data not shown). Leaching losses also tended to increase with increasing extract loading; however, losses represented only about 20% of the initial weight gain for both the synthetic and natural hinokitiol. These results suggested that the majority of the extract remained in the blocks**.** Leaching losses from blocks treated with the sodium salt of hinokitiol, however, were similar to the initial weight gains, suggesting that most of the extract was lost during the leaching period.

Table 3. Weight Losses in Poplar Blocks Treated with Various Concentrations of Natural or Synthetic Hinokitiol or its Sodium Salt and Exposed to Decay Fungi in an AWPA Standard E10 Soil Block Test

Sample	Conc. mg/mL	Wood Weight Loss *(%)*			
		T. versicolor		*G. trabeum*	
		Nonleached	Leached	Nonleached	Leached
Natural Hinokitiol	5	24.86 (13.19)	42.92 (11.61)	70.13 (5.28)	68.40 (2.64)
	10	13.17 (2.96)	22.31 (9.90)	6.52 (2.82)	45.75 (6.23)
	20	1.31 (0.72)	4.08 (2.32)	2.80 (0.76)	3.08 (0.97)
	40	3.01 (0.18)	3.64 (0.81)	2.33 (0.52)	3.48 (0.71)
Synthetic Hinokitiol	5	18.00 (16.64)	37.79 (6.38)	65.31 (3.37)	53.17 (8.99)
	10	13.58 (3.89)	29.82 (17.87)	10.60 (2.87)	48.66 (7.83)
	20	1.07 (0.65)	4.95 (3.44)	2.06 (1.21)	4.43 (2.79)
	40	2.71 (0.72)	2.05 (0.29)	2.81 (0.52)	2.32 (0.22)
Hinokitiol Na	5	15.13 (12.85)	68.70 (7.41)	46.04 (15.05)	61.27 (5.41)
	10	13.22 (6.71)	67.94 (5.99)	9.70 (5.42)	59.22 (6.02)
	20	0.46 (0.95)	70.71 (5.02)	1.63 (0.34)	60.17 (10.37)
	40	3.45 (0.50)	67.17 (7.28)	3.18 (0.76)	56.52 (6.69)
Ethanol	0	77.84 (4.08)	70.70 (5.02)	67.38 (2.36)	60.17 (10.38)
Water	0	73.08 (3.50)	63.16 (4.10)	66.39 (3.03)	68.88 (4.13)
None	--	70.45 (10.90)	70.66 (9.85)	61.84 (7.69)	64.54 (3.90)
Values represent means of 5 replicates, while numbers in the parentheses are one standard deviation.					

Weight losses for untreated poplar as well as poplar treated with either water or ethanol ranged from 60.2 to 77.8% (Table 3). These results indicate that conditions were suitable for an aggressive fungal attack. Weight losses in the control blocks tended to be higher in the blocks exposed to the white rot fungus (*T. versicolor*). This finding is consistent with the ability of white rot fungi to utilize a higher percentage of the total wood mass, as well as a tendency for white rot fungi to be more aggressive on hardwoods.

Weight losses generally decreased in non-leached blocks with an increasing extract concentration for all three materials, and both fungi tended to be inhibited at concentrations of 20 or 40 mg/mL of extract. The results with natural hinokitiol are consistent with those from previous studies (Inamori *et al.* 2000), and there were no

major differences in weight losses between synthetic and natural hinokitiol. These results indicate that synthetic hinokitiol is a suitable substitute for naturally produced material. Weight losses in non-leached blocks treated with 5 mg/mL of the sodium salt of hinokitiol tended to be lower than those found with the same treatment level of the synthetic or natural hinokitiol. The higher water solubility of the sodium salt may have increased the ability of this extract to affect fungal activity.

Weight losses in blocks treated with either the synthetic or natural hinokitiol and leached prior to fungal exposure tended to be slightly higher than those found in non-leached blocks, but the treatment remained effective when it was applied at concentrations of 20 or 40 mg/mL. A slightly higher susceptibility to a fungal attack is consistent with the gradual leaching of extractives such as hinokitiol from western red cedar in outdoor exposures (Chedgy *et al.* 2007a). Weight losses associated with blocks treated with sodium salt of hinokitiol and then leached were similar to those found with the non-treated controls. Weight measurements before and after leaching suggested that nearly all of the extract was lost in leaching, and the fungal weight loss results confirm the loss of resistance to fungal attack. These results indicate that the sodium salt was highly susceptible to leaching and would not be an effective wood protectant. While the ability to solubilize a system in water is an attractive feature, the system must then become resistant to subsequent loss once the wood has dried. The weight loss results with the sodium salt of hinokitiol suggest that further formulation studies will be required to produce an acceptable water-based system with this extract. Of paramount importance will be developing formulations that are water soluble but retain the ability to interact with the wood to become fixed or immobilized after treatment while remaining bioactive against fungi.

CONCLUSIONS

1. Natural and synthetic hinokitiol provided similar levels of protection against both mold and decay fungi.
2. Modifying hinokitiol to produce a water soluble salt did not affect efficacy; however, the salt was highly susceptible to leaching, making it ineffective against decay fungi.
3. The development of water-based hinokitiol systems will require further formulation research to produce leach-resistant formulations.

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