AN INTEGRATED APPROACH USING *Bacillus subtilis* B26 AND ESSENTIAL OILS TO LIMIT FUNGAL DISCOLORATION OF WOOD

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Bacillus subtilis and essential oils have been explored separately for their ability to limit colonization by wood stain and mold fungi, but neither approach has been completely effective. One alternative strategy would be to combine the bacterial biocontrol with one or more natural products extracts. In this report, the ability of combinations of *B. subtilis* B26 and 20 essential oils to limit fungal stain was explored on Douglas-fir sapwood wafers under controlled laboratory conditions. A number of extracts markedly improved the anti-fungal activity of *B. subtilis* B26, including 0.25% myrtlewood oil, 0.5% orange oil, 0.5% lime oil, and 1% Leyland cypress needles oil, which yielded improvements by 2 to 8 times. However, none of the combinations completely protected the wood from fungal attack. The results illustrate the difficulties associated with controlling the diverse array of organisms that can colonize freshly cut wood.

Keywords: Bacillus subtilis; Essential oils; Antifungal activity; Wood stain; Biological control; Ophiostoma perfectum; Trichoderma spp.*; Aspergillus niger*

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INTRODUCTION

Chemical treatments have long been used to protect lumber from biological discoloration during storage, transportation, and until the wood dries below 30% moisture content (Scheffer and Lindgren 1940; Kreber and Morrell 1993). While these treatments are effective, increasing public sensitivity to chemical usage has encouraged the development of less broadly toxic molecules. Despite these developments, there is still a widespread desire to completely avoid synthetic pesticides and use either natural products or biological control agents to protect wood. Three approaches that have been explored for fungal control are the use of natural products extracts from a range of plants (Kartal *et al*. 2006; Senhaji *et al.*, 2005; Šegvić Klarić *et al.*, 2007; Wang *et al.*, 2011), the use of the microorganisms to inhibit the activity of stain fungi and molds, and chemical modification of the wood (Habu *et al.* 2006). Results of laboratory trials with these systems often appear promising, but they then fail when challenged with a broader range of target organisms under field conditions. Combining natural products with selected

biological control agents may provide a novel approach for controlling microorganisms attacking wood.

Microbial wood protection has been studied for several decades (Bruce *et al*. 1991; Freitag *et al.* 1991; Benjamin 2003; Fravel 2005). Among the many possible organisms that could be used, *B. subtilis* has been heavily evaluated in the agricultural and food preservation applications, but it has also been evaluated for wood protection (Kreber and Morrell 1993). This bacterium produces a number of antifungal compounds including bacitracin, iturins, botrycidin, and subtilin (Zuber *et al*. 1993), and has shown great potential for inhibiting germination and growth of a large number of fungi, including species of *Aspergillus*, *Penicillium* (Munimbazi and Bullerman 1998), and yeasts (Thimon *et al*. 1995; Munimbazi and Bullerman 1998). Li and Chang (2008) found good fungal control in petri dish tests with *B. subtilis* B26, but limited effectiveness on birch lumber. These results illustrate the difficulty with using biological organisms as the sole protectant and advocates for a more holistic approach that incorporates multiple strategies to address the diverse array of organisms that can colonize freshly sawn lumber.

Combining low levels of natural products extracts with application of a *B. subtilis* isolate may provide a method for overcoming these limitations. In this report, the potential for using extracts from 20 different plants to enhance the activity of *B. subtilis* B26 was evaluated in both agar plate tests and on sapwood blocks.

MATERIALS AND METHODS

Microorganism Culture

B. subtilis B26 strain (B26), isolated from soil around birch trees (*Betula* sp.) in Inner Mongolia (People's Republic of China), was maintained on beef peptone slants (3 g beef extracts, 10 g peptone, 5 g NaCl, and 20 g agar per L water) and stored at 4 ˚C until use.

The test fungi, *Aspergillus niger*, *Trichoderma* spp., and *Ophiostoma perfectum*, were grown on 1.5% malt extract agar and stored at the wood preservation lab of the Wood Science and Engineering department in Oregon State University.

Essential Oils

A total of 20 essential oils obtained from various sources were evaluated in this study (Table 1). The oils were steam distilled from freshly ground materials and were refrigerated at 4 ˚C until needed.

The Consistency Test between *B. subtilis* B26 and Essential Oils

Beef peptone agar was allowed to cool to 50 ˚C before sterile-filtered ethanol dilutions of the various essential oils were added. Then the mixture was poured into plastic petri dishes and allowed to cool. Extracts were assessed at 0.1, 0.25, 0.75, or 1.00 % (wt/wt). A 5 mm disc cut from the actively growing edge of a culture of strain B26 was placed at the center of each plate and incubated at 30 ˚C for 5 days. Each extract was evaluated on 5 petri plates, and control plates were evaluated with only ethanol as the

additive. The diameter of bacterial growth around the original agar plug was then assessed and used as a measure of the compatibility of the B26 strain with the extracts.

Table 1. Characteristics of Essential Oils Evaluated for Protecting Wood against Fungal Stain and Mold

Name	Source	Plant species
Cinnamon oil	Foliage	*Cinnamomum* spp.
Noble fir oil (a)	Foliage	*Abies procera* Rehd.
Noble fir oil (b)	Foliage	*A. procera* Rehd.
Isothymol (carvacrol)	Foliage	*Chamaecyparis nootkatensis* (D.Don) Spach
Redwood leaf oil	Foliage	*Sequoia sempervirens* (D.Don) Endl.
Myrtlewood	Heartwood	*Umbellularia californica* (Hook & Arn) Nutt.
Western Juniper needles	Foliage	*Juniperus occidentalis*
Eucalyptus oil	Foliage	*Eucalyptus* spp.
Lodgepole pine	Heartwood	*Pinus contorta* (Dougl)
Alaska cedar leaf oil	Foliage	*C. nootkatensis* (D.Don) Spach
Bitter orange	Fruit	Unknown
Douglas-fir oil	Foliage	*Pseudotsuga menziesii* (Mirb.) Franco
Monterey cypress foliage	Foliage	*Cupressus macrocarpa* Hartweg
Lime oil	Fruit	*Citrus* x *aurantiifolia* (Christm.) Swingle
Incense cedar	Heartwood	*Libocedrus decurrens* Torr.
Sequoia oil	Heartwood	*Sequoia gigantea* (Lindl) Decne.
Orange oil	Fruit	*Citrus* x *sinensis* (L.) *Osbeck*
Leyland Cypress needles	Foliage	*Cupressus x leylandii* (syn. × *Cupressocyparis leylandii*, *Callitropsis* × *leylandii*),
Lemon oil	Fruit	*Citrus* x *limon* (L.) Burm.
Lemon grass oil	Foliage	*Cymbopogon* spp.

Antifungal Activity of *B. subtilis* B26 with Essential Oils

Freshly sawn Douglas fir sapwood lumber (*Pseudotsuga menziesii* (Mirb.) Franco) was obtained from the sawmill and cut into 5 by 10 by 30 mm long blocks that were then frozen until needed. Test blocks were allowed to thaw before use, then dipped for 30 seconds in an excess of a given dilution of an essential oil. The blocks were placed on trays in a sterile transfer hood to allow the ethanol to dissipate.

Glass petri dishes containing a plastic mesh on top of three 90 mm diameter Whatman #1 filter paper discs served as the exposure chamber. The filter paper was wetted with 5 ml of distilled water before the plates were sterilized by heating for 20 minutes at 121 ˚C. Five Douglas-fir sapwood blocks from a given treatment were then placed on top of the screen in each plate. The blocks were either left alone or sprayed with the bacterial suspension, then sprayed with a spore/hyphal suspension of a given test fungus immediately after bacterial application, 1 day later or 3 days later. The delay was intended to determine if giving the bacteria time to colonize the substrate would make them more active against the test fungus.

The fungal inoculum was prepared by growing the fungus on 0.5 % malt extract at 27˚C for 24 hours, then collecting the resulting spores and mycelium by vacuum filtration. This mixture was resuspended in sterile distilled water, macerated briefly in a blender and then placed in a spray bottle for application to the wood. The spore/hyphal fragment concentration was adjusted to a 10^7 colony forming units/mL prior to use. Each extract/bacterial incubation time/fungal treatment was replicated on 20 blocks. The plates were sealed with a paraffin wax film to retard drying and incubated at room temperature (20 to 23 ˚C) for 30 weeks. The degree of discoloration on each sample was assessed visually on a scale from 0 (no discoloration) to 100 (complete discoloration). Blocks dipped in distilled water or ethanol were used as untreated controls, while blocks treated in 1.0 % propiconazole were used as positive controls.

RESULTS AND DISCUSSION

The Consistency Test between *Bacillus subtilis* B26 and Essential Oils

Most of the essential oils were not broadly toxic to *B. subtilis* B26 except cinnamon oil, isothymol, and lemon grass oil which killed the bacterium at a concentration of 0.1% (Figures 1-3). *Bacillus subtilis* B26 growth increased when incubated together with redwood leaf oil, Alaska cedar leaf oil, bitter orange oil, Douglas-fir oil, lime oil, sequoia oil, or Leyland cypress needle oil. The remaining materials had no effect on *B. subtilis* B26 at the concentrations tested. Clearly, oils that negatively affect the bioprotectant will not be suitable for integrated protection; however, those that either have no effect or that enhance growth would be suitable and merit further testing.

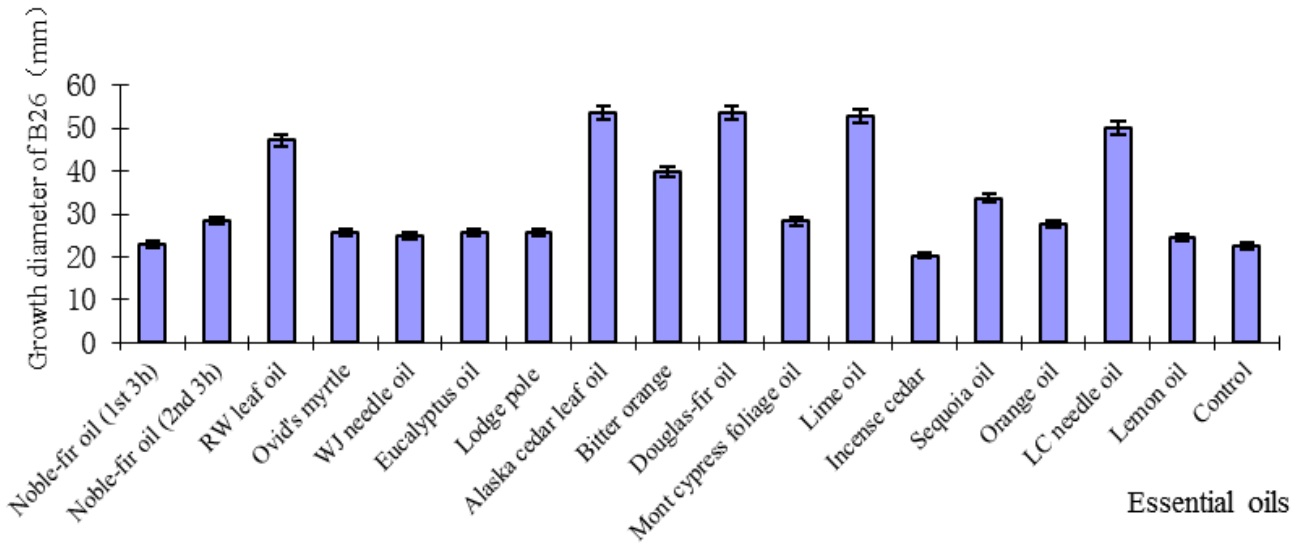

Fig. 1. Growth of *B. subtilis* B26 in the presence of a 0.25 % of a given essential oil

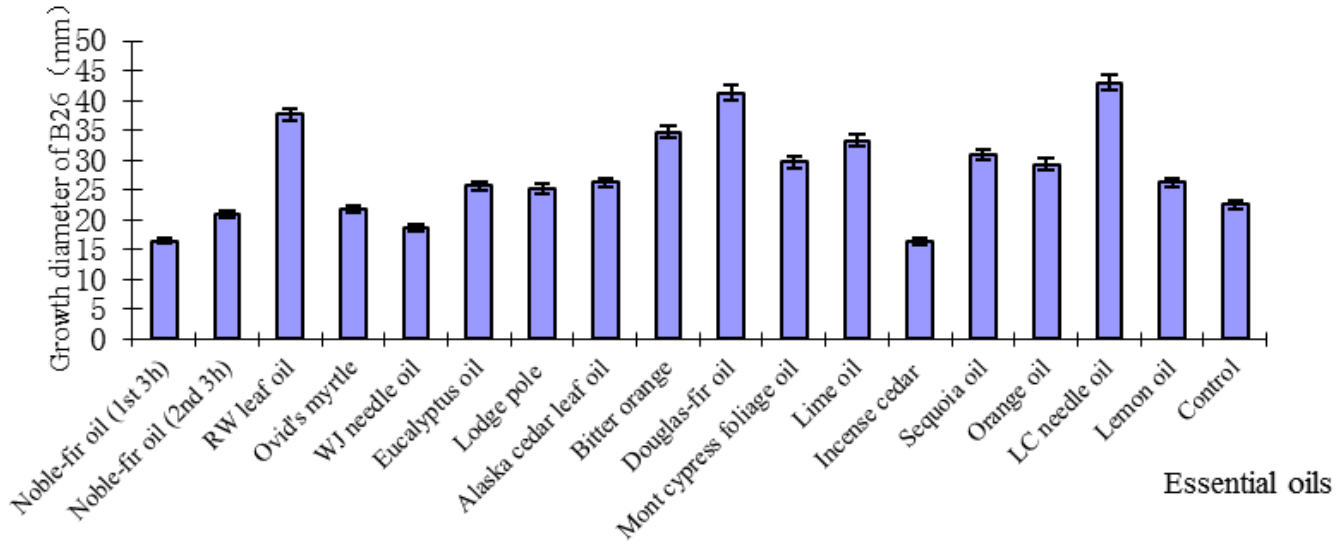

Fig. 2. Growth of *B. subtilis* B26 in the presence of 0.50 % of a given essential oil

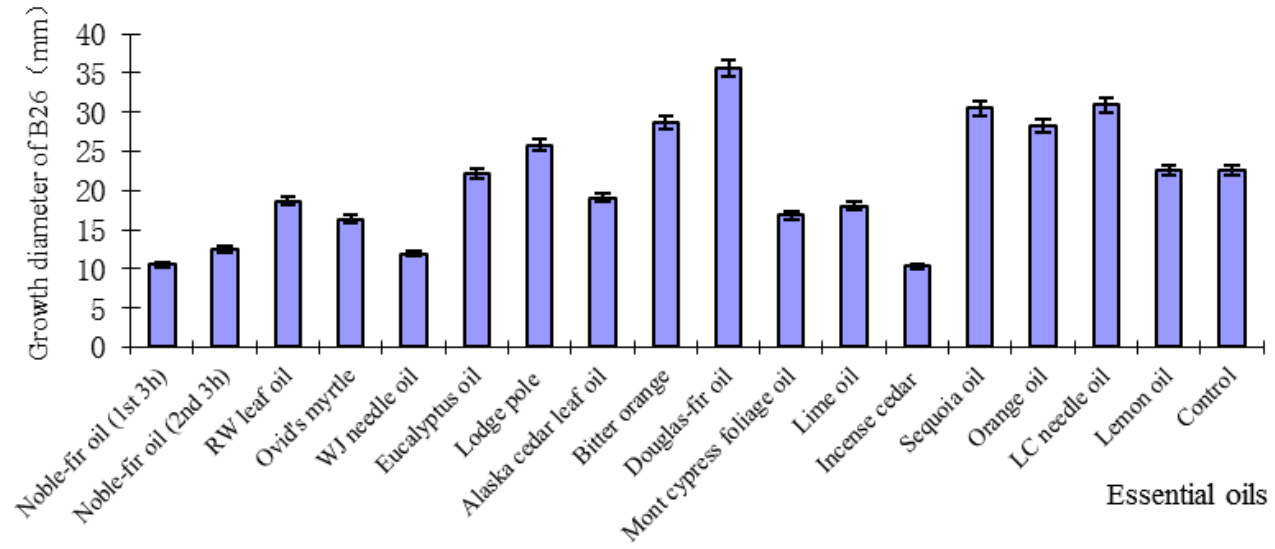

Fig. 3. Growth of *B. subtilis* B26 in the presence of 1.00 % of a given essential oil.

Antifungal Activity of *B. subtilis* B26 with Essential Oils

Ideally, the presence of the extract should produce some inhibition of the test fungus and the addition of the bacterium should produce complete inhibition of fungal growth and discoloration. Delaying the time between introduction of the bacteria and the fungus should allow the bacteria to colonize the substrate and potentially utilize nutrients necessary for initiation of fungal growth. It might also provide some time for the bacteria to begin to produce antibiotic compounds. In general, however, increasing the colonization period for the bacterium should result in improved protection.

Our results indicated that lemon grass oil, cinnamon oil, isothymol, and lodgepole pine oil could completely inhibit *Trichoderma* spp. on wood. The remaining extracts were associated with some discoloration. In some cases, the addition of *B. subtilis* B26 was associated with a slight increase in discoloration (Table 2).

The addition of *B. subtilis* B26 sometimes produced a decline in degree of discoloration that increased with time between application of the bacteria and the stain fungi. Examples of this trend included blocks treated with redwood leaf oil, Alaska cedar oil, bitter orange, Douglas-fir oil, sequoia oil, orange oil, and Leyland cypress oil. These results suggest that the combination of the natural product extract and the bacteria could produce more effective fungal control.

The essential oils had much less effect on discoloration by *O. perfectum* on wood (Table 3). Only noble fir oil (b) completely inhibited fungal growth, while the remaining treatments were associated with 15 to 70 % discoloration. This fungus is adapted for growth on sapwood and these results clearly illustrate this ability. Treatment with essential oils and *B. subtilis* B26 produced variable results. For example, cinnamon oil and the bacteria resulted in complete inhibition at both levels tested, while many other treatments were associated with reductions in the degree of discoloration observed. Delaying the time between addition of the bacteria and the fungus was sometimes associated with reductions in discoloration, but the trends were inconsistent. In general, however, there was little difference in degree of discoloration on samples treated with extracts and the bacterium, then immediately inoculated with the fungus compared with those where the fungal application was delayed.

B. subtilis B26 was associated with reduced discoloration when used in combination with noble fir oil (b), lime oil, orange oil, or lemon oil against *A. niger* (Table 4). In a number of other cases, the degree of discoloration varied widely with time of introduction of the stain fungus, suggesting that the combination had little effect on fungal growth. The results suggest that combining essential oils and *B. subtilis* produced no consistent improvement in *A. niger* control.

Further studies are planned using extracts from different plant sources in combination with *B. subtilis* B26.

Table 2. Effect of *B. subtilis* B26 and Essential Oils on Degree of Discoloration on Douglas-fir Sapwood Wafers Inoculated with *Trichoderma* spp.

Essential oils	Conc.(v/v)	Degree of Fungal Discoloration (%)			
		Trichoderma spp.	*Trichoderma* spp. + B26	*Trichoderma* spp.1 *+ B26	*Trichoderma* spp.3 * + B26
Noble-fir oil (a)	0.25%	2	5	10	15
Noble-fir oil (b)	0.25%	25	40	30	50
Redwood leaf oil	0.25%	35	20	1	5
Myrtlewood oil	0.25%	35	15	65	1
WJ needle oil	0.25%	5	30	5	2
Eucalyptus oil	0.50%	20	40	15	10
Lodgepole pine oil	0.50%	0	5	15	30
Alaska cedar leaf oil	0.50%	60	30	15	5
Bitter orange oil	0.50%	75	25	10	2
Douglas-fir oil	0.50%	25	15	15	1
Mont' cypress foliage oil	0.50%	5	10	15	10
Lime oil	0.50%	45	20	70	1
Sequoia oil	0.50%	30	10	15	10
Orange oil	0.50%	15	2	1	0
LC needle oil	1.00%	30	15	5	5
Lemon oil	1.00%	10	65	50	5
Control-water		100	-	-	-
Control-ethanol	1.00%	100	-	-	-
Control-propiconazole	1.00%	0	-	-	-

* 1 and 3 stand for time interval (days) between inoculation with B. *subtilis* B26 and *Trichoderma* spp. Values represent average percentages for 20 blocks per treatment combination.

Table 3. Effect of *B. subtilis* B26 and Essential Oils on Degree of Discoloration on Douglas-fir Sapwood Wafers Inoculated with *O. perfectum*

Essential oils	Conc.(v/v)	Degree of Fungal Discoloration (%)			
		O. perfectum	*O. perfectum* + B26	*O. perfectum* 1 * + B26	*O. perfectum* 3 * + B26
Noble-fir oil (a)	0.25%	0	35	30	15
Noble-fir oil (b)	0.50%	30	60	10	30
Redwood leaf oil	0.25%	30	90	30	20
Myrtlewood oil	0.25%	45	30	60	5
WJ needle oil	0.25%	65	80	25	15
Eucalyptus oil	0.50%	45	40	40	25
Lodgepole pine oil	0.50%	40	25	45	5
Alaska cedar leaf oil	0.50%	50	60	20	20
Bitter orange oil	0.50%	20	50	25	0
Douglas-fir oil	0.50%	70	40	10	0
Mont' cypress foliage oil	0.50%	15	70	45	20
Lime oil	0.50%	20	35	15	0
Sequoia oil	0.50%	20	10	30	5
Orange oil	0.50%	5	5	30	5
LC needle oil	1.00%	90	70	30	5
Lemon oil	1.00%	45	70	45	40
Control-water		90			
Control-ethanol	1.00%	80			
Control-propiconazole	1.00%	0			

* 1 and 3 stand for time interval (days) between inoculation with B. *subtilis* B26 and *O. perfectum*. Values represent average percentages for 20 blocks per treatment combination.

Table 4. Effect of *B. subtilis* B26 and Essential Oils on Degree of Discoloration on Douglas-fir Sapwood Wafers Inoculated with *A. niger*

Essential oils	Conc.(v/v)	Degree of Fungal Discoloration (%)			
		A. niger	*A. niger* + B26	*A. niger* 1 * + B26	*A. niger* 3 * + B26
Noble-fir oil (a)	0.25%	20	2	10	10
Noble-fir oil (b)	0.25%	30	15	10	2
Myrtlewood oil	0.25%	50	75	60	0
Redwood leaf oil	0.50%	15	0	15	10
Alaska cedar leaf oil	0.50%	80	20	90	10
Bitter orange oil	0.50%	90	20	50	40
Lime oil	0.50%	85	65	5	0
Orange oil	0.50%	5	5	5	0
LC needle oil	1.00%	35	65	40	5
Lemon oil	1.00%	70	55	45	25
Control-water		100			
Control-ethanol	1.00%	95			
Control-propiconazole	1.00%	0			

* 1 and 3 stand for time interval (days) between inoculation with B. *subtilis* B26 and *A. niger*. Values represent average percentages for 20 blocks per treatment combination.

CONCLUSIONS

Myrtlewood oil (0.25 %), orange oil (0.5 %), lime oil (0.5 %), and Leyland cypress needle oil (1.0%) all appeared to act synergistically with *B. subtilis* B26 to protect wood against fungal attack. However, none of the treatments completely protected Douglas fir sapwood blocks against molds and stain fungi as well as the chemical fungicide propiconazole. While it might be possible to explore the efficacy of higher concentrations of individual extract components, this would likely reduce the value of these products as natural treatments.

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