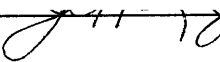


AN ABSTRACT OF THE THESIS OF

Bernhard Kreber for the degree of Master of Science in Forest Products presented on November, 8, 1991 .

Title: Bioprotection of Unseasoned Ponderosa Pine Lumber from Microbial Discoloration .

Abstract approved: Signature redacted for privacy.
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The potential of 9 bacterial and 6 fungal isolates to protect wood against wood staining microorganisms was studied using small sapwood samples of unseasoned Ponderosa pine [Pinus ponderosa Laws] sapwood. Bacillus subtilis Cohn, isolate 733 A , Gliocladium virens J.H. Miller, J.E. Giddens & A.A. Foster isolate H 3 and Pseudomonas putida Migula isolate A-12 were identified as promising biological agents.

The effect of media on the ability of B. subtilis # 733 A and B. subtilis ATCC 6633 to protect Ponderosa pine specimens from microbial discoloration was evaluated. A media preference was demonstrated with each of the species tested, which enhanced bioprotectant capability. B. subtilis # 733 A prevented fungal stain on Ponderosa pine to a greater degree than B. subtilis ATCC 6633.

Extracts from Ponderosa pine specimens inoculated with B. subtilis # 733 A and/or fungal stain, exhibited no evidence of antibiosis against selected stain fungi and thus

yielded little information on the modes of action of the biological agent.

In situ observations on spore germination of Aspergillus niger van Tieghe isolate BOB in the presence of B. subtilis 733 A using a designed incubation chamber suggested that prior bacterial colonization of the wood surface did not inhibit germination of A. niger conidia.

These results indicate that bioprotection is affected by a variety of factors including wood species, temperature, nutritional status, and isolate source. Considerable research will be necessary to successfully develop effective bioprotectants against fungal stain.

Bioprotection of Unseasoned Ponderosa Pine
Lumber from Microbial Discoloration

by

Bernhard Kreber

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

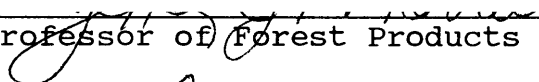
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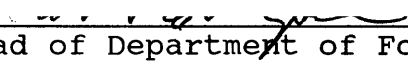
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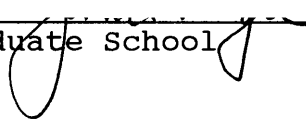
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discolored wood is not accepted by customers and its value is reduced. Annual economic losses due to discolored lumber have been conservatively estimated at about \$ 10,000,000 [Scheffer, 1973].

R. Hartig [1878] first recognized the nature of sapwood staining and attributed it to a fungus called Xenodochus ligniperda. In 1903, H. von Schrenk studied blue-stain of Ponderosa pine in North America, which he attributed to Ceratocystis pilifera. Münch [1907] and Lagerberg [1927] conducted further pioneering work on stain fungi, noting the ability of several fungal species to stain wood. Scheffer and Lindgren [1940], described in a very comprehensive study, biological stain characteristics and their control. Verrall [1941], Findlay [1959], Liese and Schmid [1961, 1964], Schmid and Liese [1965] and Zink and Fengel [1988, 1989, 1990] all significantly contributed to our current understanding of biological discolorations and their prevention.

Protection of unseasoned lumber from wood discoloration during storage and transportation has traditionally been accomplished with chlorinated phenates [CP] prophylactically applied in a dip or spray treatment. CP's have been extensively used since 1930 and provide excellent low cost protection against stain and mold fungi. However, potential carcinogenic effects and the risk of CP contaminations have raised considerable concerns [BC, Ministry of Environment,

1983]. As a result, the use of chlorinated phenates as prophylactic treatments has severely been regulated [e.g. U.S., Canada] or completely banned [e.g. Sweden, Germany]. This action has led to an intensive search for safer chemicals to replace CP's [Hulme and Thomas, 1979; Cserjesi and Johnson, 1982; Drysdale and Preston, 1982; Eslyn and Cassens, 1983; Miller and Morrell, 1989; Miller et al. 1989]. However, recently formulated antistain fungicides may also create some human health problems and environmental concerns [Drysdale 1987]. Many newer formulations are also less effective and more expensive when compared with chlorinated phenates. Thus, there still is a need for improved antistain treatments [Drysdale, 1987].

Increasing public awareness of potential hazards of wood preservatives [e.g. antistain fungicides], combined with a decreasing acceptance of the use of these chemicals [Cox, 1991], suggests that new approaches for protecting unseasoned lumber during storage and transportation are required.

Although the degree of stain can be reduced by good handling practices which include proper piling, sanitation and inventory practices, wood remains susceptible to stain as long as wood moisture is above the fiber saturation point. Currently, the only effective non-chemical method for preventing wood discoloration is to dry freshly cut lumber below the fiber saturation point as quickly as possible,

usually through kiln-drying. This approach is often impractical because of the energy involved. Kiln-dried lumber may also take up moisture during storage and transportation to again become susceptible to wood discolorations [Williams, 1990].

One alternative to chemical antistain protection which is actively being explored is termed biological protection. Biological protection or control has opened new avenues in agriculture to combat plant pathogens or post harvest diseases [Baker and Cook, 1974; Cook and Baker, 1983]. Research on biological control of plant diseases has included programs to limit Take-All of wheat [Gaeumannomyces graminis (Sacc)Arx & D. Olivier] [Weller and Cook, 1983; Thomashow et al. 1990], Damping-off [Howell, 1981; Elad and Chet, 1987] and a variety of post harvest diseases [Pusey and Wilson 1984; Wilson and Pusey, 1985; Janisiewicz, 1988; Wilson et al. 1989].

Bioprotection of wood products has also been studied for preventing wood decay or discoloration. Strategies in agriculture and postharvest systems differ from those required for protection of wood products. The best known and only commercially successful example of biological wood protection is the use of Phanerochaete gigantea (Fr.:Fr.) Rattan to prevent root and butt rot in European Scots pine [Pinus sylvestris L.] caused by Heterobasidion annosum (Fr.) Bref. [Barton, 1961; Rishbeth, 1963].

Production of lumber for domestic and export markets is heavily dependent on prophylactic chemical treatments to prevent wood discoloration. The uncertainty of the future availability of chemicals has encouraged this study to screen for biological agents capable of inhibiting fungal stain. Identifying effective bioprotectants and understanding the modes of action may reduce chemical dependence, decrease economic losses and allay some environmental concerns associated with wood processing industries.

In this study four objectives were investigated:

- * Identify and evaluate the ability of selected microorganisms to protect Ponderosa pine sapwood from biological stain.

- * Evaluate the effects of media on the effectiveness of bioprotectants.

- * Assess the fungitoxicity of extracts harvested from Ponderosa pine specimens inoculated with a selected bioprotectant and/or a stain fungal suspension on selected mold and stain fungi.

- * Assess the effects of a selected bioprotectant on spore germination of a mold fungus.

2.0. Identify and Evaluate the Ability of
Selected Microorganisms to Protect Ponderosa
Pine Sapwood from Biological Stain

2.1. Concepts and requirements in biocontrol and
bioprotection

2.1.1. Introduction

Protection of wood against degrading organisms under conditions where entry of moisture into the wood can not be controlled has generally been accomplished by chemical treatments [Graham, 1973]. Increasing public awareness about potential hazards of chemicals has resulted in restrictions on the use of some wood preservatives and initiated a search for safer chemicals or non-chemical alternatives.

The use of microorganisms to combat plant pathogens or post harvest diseases has been extensively studied in agriculture and represents a desirable non-chemical alternative. This strategy, termed biological control or biocontrol, focuses on limiting damage to crops or restraining agricultural and horticultural pests. In most cases, however, crop protection employing biocontrol agents is targeted against a single species or a narrow range of pathogens and the period of protection rarely extends over more than one single growing season. Two formulations of a

biological agent, G. virens isolate GL-21, have recently been approved by the Environmental Protection Agency [EPA] for application against damping-off caused by Pythium sp. and Rhizoctonia sp. on vegetable or ornamental bedding plants in the greenhouse [Anonymous, 1991].

Microorganisms have also been employed for preventing biodeterioration of wood products. In wood systems, however, substrate protection is the ultimate goal of biological wood protection since significant damage may already occur in incipient stages of fungal infestation [Wilcox, 1978]. Therefore, the strategy has more accurately been termed bioprotection instead of biocontrol as is used in agriculture.

Furthermore, wood bioprotection differs from agricultural biocontrol strategies in the long protective period required, the presence of a variety of target species which are capable of damaging the wood substrate and the fact that failures in wood systems, particularly in structural commodities, are not acceptable.

Thus, an ideal wood bioprotectant will rapidly colonize and occupy a vacant resource [wood] and change the substrate sufficiently to prevent colonization by other competing microorganisms such as decay and stain fungi without harming the wood. While this strategy involves early arrival and the utilization of readily available, simple nutrients, combative interaction is also likely to occur, especially

when long term protection is required. Combative competition resulting in successful defense of the resource or secondary resource capture may involve antibiosis [Gottlieb and Shaw, 1970; Fravel, 1988], lysis [Carter and Lockwood, 1957] or parasitism [Mitchell and Alexander, 1963]. Although combative interactions have been extensively studied in culture, little is known about these interactions within the wood substrate.

2.1.2. Potential Applications for Bioprotection of Wood Products

Possible applications for bioprotection of wood products include preventing decay, eliminating incipient decay and preventing fungal discoloration in unseasoned wood. However, research on bioprotection of wood has primarily focused on preventing colonization by decay fungi [Ricard, 1966; Richard and Bollen, 1967; Toole, 1971; Ricard, 1976; Bruce and King, 1983; Bruce and King, 1986 a+b;] and eliminating decay fungi at the incipient stage of attack [Ricard et al., 1969; Morris and Dickinson, 1981; Highley, 1989]. The majority of this research has utilized Trichoderma harzianum Rifai, T. polysporum (Link.:Pers.) Rifai or Scytalidium Strain FY as potential bioprotectants.

2.1.3. Bioprotection of Wood Decay

Although bioprotectants evaluated for decay protection or incipient decay prevention have shown promising results in culture [cross plating on agar], studies utilizing wood substrates have highlighted three major problems with the application of bioprotectants against wood decay fungi:

- * the inability of bioprotectants to completely colonize the substrate
- * an inability to provide long term protection against wood degrading fungi
- * the inability to effectively inhibit the wide range of target wood decay species.

The inability of bioprotectants to completely colonize the substrate, especially when competing, non-decay fungi are present is a major concern [Graham ,1973; Bruce and King, 1986 a+b]. However, it is interesting to note that wood successfully colonized by Trichoderma spp. exhibited decay resistance in soil block tests [Bruce et al., 1989] and remained protected 7 years after inoculation [Bruce et al., 1990]. This long term protection demonstrates that bioprotection is feasible when colonization of the wood substrate occurs.

The addition of nutrients that favor growth of the bioprotectant has been considered to assist colonization of the bioprotectant [Morrell, 1990], as has the application of

low levels of diffusible biocides to reduce competition and improve colonization [Dawson-Andoh and Morrell, In Press], but neither of these strategies have been extensively tested. Although the latter approach involves some chemical treatments, the use of small amounts of chemicals may be justified by the improved levels of bioprotectant colonization.

The inability to provide long term protection against wood degrading fungi poses a major challenge for protecting wood products for their service life. Long term wood protection [up to 40 years] requires the production of chemically stable, toxic compounds [antibiotics] that remain active within the substrate for many years. Production of survival structures which revitalize combative action once a target decay fungus has entered the wood substrate is also desirable. Although several studies have suggested production of antibiotics as the probable mode of action by which bioprotectants inhibit decay organisms [Klingström et al., 1973; Stillwell et al.], the activity and stability of these compounds within the wood remains questionable [Morris and Dickinson, 1986; Highley, 1989].

The inability to effectively inhibit the wide range of target species that decay wood may result in exclusion of only part of the microflora present in the wood substrate. Wood species such as Douglas-fir or Southern pine can be infested by a variety of brown-, white-, and soft rot fungi

[Eslyn, 1970; Graham and Corden, 1980; Zabel et al., 1980, 1985; Wang and Zabel, 1990]. Thus, bioprotectants employed in these systems must be effective against a broad spectrum of decay fungi. Although bioprotection has been demonstrated against selected decay fungi, a broad spectrum inhibition of economically important decay fungi by a bioprotectant has not yet been demonstrated [Cease et. al., 1989; Highley, 1989, Morrell and Sexton, 1990].

2.1.4. Bioprotection of Unseasoned Sapwood from Microbial Stain

Research on bioprotection against wood discoloration has received much less attention, although the short period required for prevention of wood staining would seem to have the highest potential for successful application of bioprotection.

Debarking and sawing expose wood to colonization by a wide range of microorganisms [Butcher, 1968]. The topography of the wood surface produces a variety of microhabitats. Small checks or erupted fibers can provide localized zones where water and nutrients are more accessible and can protect the fungus from ultraviolet light. For instance, Ceratocystis spp. tends to invade the wood substrate through tangential surfaces protected from UV light and desiccation [Dowding, 1970]. Colonization of a

substrate is generally initiated through deposition of air and rainborne spores on the wood surface. These spores germinate and penetrate the wood surface within hours when conditions such as temperature and moisture are favorable. Biocides for prevention of fungal stain must therefore be applied within 24 hours, otherwise stain fungi may penetrate the wood substrate below the depth commonly reached with fungicidal dips or sprays.

Bioprotection against microbial stain may also require introduction of a bioprotectant on the wood substrate immediately following sawing. The function of a bioprotectant on unseasoned sapwood is similar to foliar disease protection in that surface exclusion plays a major role in bioprotectant performance. The surface of freshly sawn boards has limited quantities of readily available nutrients released by rupturing of the ray parenchyma and a great variety of microorganisms actively competing for these substrates. Furthermore, fluctuating environmental conditions [temperature, UV-light, wood moisture] stress microorganisms on the wood surface. Ideally a bioprotectant would penetrate the wood surface to inhibit competing microbes; however, the formation of an intact surface barrier by the bioprotectant might be sufficient if the biological agent is adapted to environmental stresses such as desiccation or UV-light and can survive for several months on the wood surface.

2.1.5. Literature Review - Bioprotection of
Unseasoned Sapwood from Microbial
Discoloration

Bioprotection of wood discolorations has been significantly influenced by two scientists, Benko and Seifert who separately performed a series of detailed, large scale screening trials of bacterial and fungal bioprotectants.

Benko [1986] evaluated the potential of over 100 fungi belonging to the subclasses of Basidiomycotina, Ascomycotina and Deuteromycotina to inhibit Ceratocystis coerulescens [Münch] Bakshi using malt agar cross platings. Promising fungal bioprotectants belonging to the genera Gliocladium spp. and Trichoderma spp. and two mycorrhizal fungi Clitocybe geotropa [Bull.] Quel. and a unidentified strain named D 37, exhibited strong antagonistic action. The strong antagonistic ability of the latter two fungi was attributed to production of a powerful metabolite and extraction of this compound as a biocide was suggested. Benko [1987] subsequently demonstrated that extracts of fungal cultures of 2 mycorrhizal fungi were more active against C. coerulescens employing a malt agar cross plating method and pine [Pinus sylvestris L.] specimens than the active fungus. Twenty-two parasitic fungi were evaluated against C.

corulescens, Aureobasidium pullulans [deBary] Arnaud and Sclerophoma spp. [Benko and Henningson, 1986]. The white rot fungus Bjerkandera adjusta [Willd.:Fr.] Karst was highly parasitic, penetrating into the hyphae of the blue stain fungus. In addition to these well known parasitic actions, an almost complete bleaching of the hyphae of the blue stain fungus was observed. The authors suggested that enzymatic reactions, probably involving peroxidases, caused the disappearance of the dark brown or black melanin pigments deposited within the hyphal cell wall.

Benko [1988] also investigated the potential of 150 bacteria belonging to 15 different genera for their ability to inhibit fungal growth on dual culture malt agar plates. A Pseudomonas spp., later identified as P. cepacia Burkholder, exhibited strong antagonistic activity against A. pullulans, C. coerulescens and Ceratocystis spp.. In a subsequent study, P. cepacia isolate # 6253 effectively limited staining over a four month period on Pinus radiata D. Don. specimens placed onto the surface of mycelium of fungal staining organisms [Benko, 1989]. However, field trials of logs sprayed over the entire surface or only on the log ends with the bacterium produced complete protection for three weeks, after which protection gradually declined. Possible explanations for the different outcomes in laboratory and the field trials were traced to the presence of moisture

gradients in the logs and the inability of the biological agent to deeply penetrate the logs.

There have been a variety of screening trials of potential bioprotectants, but most have been performed on agar containing excess nutrients. Nutritional status of a bioprotectant and its intended target may have a significant impact on outcome. The effect of various media on the antagonistic capability of six bacteria was evaluated against Postia placenta [Fries] M. Larson et Lombard, Trametes versicolor [Fries] Quilet, C. coerulescens and Trichoderma harzianum Rifai, respectively. Yeast-dextrose-peptone-agar and sporulation agar equally supported fungal and bacterial growth, but selectively promoted bacterial production of secondary metabolites [Benko and Highley, 1990 a]. These results illustrate the significance of media in bioprotection screening experiments. Benko and Highley [1990 b] also investigated the ability of six bacteria grown in 14 different liquid media to protect Southern pine specimens. Samples were dipped into a mixture of the supernatants of six biological agents and then placed on top of mycelial mats of wood staining fungi. Bacteria grown in sporulation broth provided the best protection against wood attacking and staining fungi.

In a very detailed screening study, Seifert et al. [1987] investigated Bacillus subtilis Cohn isolate # 186 , a strain previously suggested as a potential bioprotectant by

Bernier and coworkers [1986], against 45 staining fungi. All but one of the stain fungi were sensitive to the bioprotectant, as evidenced by formation of inhibition zones, or in some cases, changes in fungal morphology in dual culture plates. Bacterial metabolites appeared to be fungistatic rather than fungitoxic. Screening trials indicated that pine specimens colonized two weeks earlier by the bioprotectant were generally less stained but fungal growth was not completely prevented. Microscopic observations suggested that incomplete colonization of the substrate was the probable reason for bioprotectant failure.

Florence et al. [1990] also studied the potential of B. subtilis to inhibit growth of Botrydiplochia theobromae Pat. on various important Indian wood species. Rubber wood [Hevea brasiliensis], a highly stain susceptible wood species, was dipped [5 sec] into a suspension of the bioprotectant and was completely protected for a two week period.

Seifert et al. [1988] screened 88 fungal strains against sapstain fungi on agar. Promising bioprotectants were then evaluated in precolonization experiments where the bioprotectant was introduced into pine [Pinus banksiana Lamb.] specimens 2 weeks prior to the stain fungi.

Acremonium strictum W. Gams isolate 314 A, Bysochlamys nivea Westling isolate 681 A, Penicillium thomii Maire isolate 655 B and Stilbella aciculoga [Ellis & Everh.] Seifert were identified as promising antistain bioprotectants.

2.1.6. Mode of Action of Stain Bioprotection

Generally three mechanisms, antibiosis, competition and parasitism appeared to be the probable modes of action for preventing wood discoloration. However the main body of bioprotection studies evaluating the potential of antifungal compounds have been performed in liquid media. Culture fluids [Croan and Highley, 1991 a] and cell-free supernatant [Croan and Highley, 1991 b; Highley *et al.*, 1991] completely protected Southern pine and Sweetgum [Liquidambar styraciflua L.] against stain and mold fungi for 8 weeks. An antibiotic, Scytalidin, produced by Scytalidium FY strain was also investigated as a potential biological agent against the mold and stain fungi prevalent on pulpwood chips in outside storage and against Graphium spp. on white pine [Pinus strobus L.] sapwood [Stranks, 1976] or on nutrient media [Stillwell *et al.*, 1973]. The authors, however, noted two different outcomes regarding protection against Graphium spp., possibly reflecting strain variations of the biological agent as well as the virulence of the blue stain fungi tested. Klingström *et al.* [1973] demonstrated wide variations in antagonistic action in 38 strains of Scytalidium spp. when tested against decay fungi and the blue stain fungus Leptographium lundbergii Lagerberg *et* Melin. Interestingly, those variations were independent of geographical source.

Although antibiosis is widely recognized as a significant mechanism by which bioprotection of fungal stain occurs, *in situ* [wood substrate] production of antibiotics has not yet been demonstrated. Antifungal compounds produced in culture fluid may also not be inhibitory to all of the important mold and stain fungi. Furthermore, stability of secondary metabolites, once introduced into the wood, particular under fluctuating environmental conditions remains unclear. For example, Scytalidin can be leached from wood samples [Morris *et al.*, 1986].

Undoubtedly, competition for space and nutrients plays an important role in successful bioprotection against stain fungi. Bacteria have been employed as potential bioprotectants because of their high growth rates which may result in a rapid exploitation of the substrate to exclude competitors. However, the bacterial bioprotectants evaluated have exhibited poor colonization properties and were unable to completely occupy the substrate [Seifert *et al.*, 1987; Benko, 1989]. Bacteria may further be unable to sequester all of the readily available nutrients, leaving nutrients available to support the growth of microbial competitors, including stain fungi.

Parasitism has been demonstrated in bioprevention of stain [Benko and Henningson, 1986; Croan and Highley, 1991 a], but organisms employed may also disfigure the wood because of their potential to decay wood or through mass

production of colored spores on the wood surface. Although decolorization of stain fungal hyphae by parasitic action has been reported [Benko and Henningson, 1986; Croan and Highley, 1991 a] the mechanism still remains unknown and its occurrence probably has relatively little practical value. Unless the mode of action of these bleaching actions can be elucidated, mycoparasitism does not seem to provide a successful strategy for bioprotection of all fungal stain.

Successful bioprotection of unseasoned sapwood from microbial stain would appear to be most likely using a culture fluid containing a competitive bioprotectant as well as toxic secondary metabolites. The presence of antimicrobial compounds and possible addition of selective nutrients may provide a "starting advantage" by inhibiting competing microbes and promoting complete substrate colonization by the bioprotectant. This "starting advantage" may persist for a sufficient time period for the wood moisture content to decrease sufficiently to limit colonization by competing mold and stain fungi.

2.1.7. The Importance of Screening Trials

The majority of bioprotection studies to identify biological agents for preventing microbial stain have employed cross platings on agar media. Although this method is simple and rapid, outcomes differ significantly from

investigations performed on wood. Evaluations performed on wood can provide immediate clues about the potential of a biological agent to inhibit fungal stain. Furthermore laboratory trials should closely resemble the conditions in the wood where numerous microorganisms are almost simultaneously competing for the same limited resource.

2.2. Material and Methods

2.2.1. Bioprotectants and Stain Fungi Tested

Fifteen bacterial and fungal species were evaluated for their ability to protect unseasoned Ponderosa pine [Pinus ponderosa Laws] lumber from microbial discoloration. These organisms were obtained from a variety of environments including wood and soil. Bioprotectants are listed in Table 2.1 with current name, strain number, source and growth media. Fungi capable of staining wood were selected from the Forest Research Laboratory culture collection [Corvallis, Oregon] and are recorded in Table 2.2 with current name and strain number. Bioprotectants were maintained on agar slant tubes employing the appropriate media [Tab. 2.1]. Stain fungi were maintained on malt agar [1.5 % malt extract, 1 % agar]. All cultures used were stored in a cold room [5°C] until needed. All chemicals used for media composition were purchased by Difco Laboratories [Detroit, MI] or Sigma

Table 2.1: Sources of organisms and media employed for maintaining stock cultures of bioprotectants evaluated on Ponderosa pine.			
Species	Strain Number	Source	Growth Media
BACTERIA			
<u>Bacillus subtilis</u>	733 A	Forintek Canada Corp., Ottawa, Canada	Nutrient Broth [NB]
<u>B. subtilis</u> Cohn	Quantum 4000 HB	J. Loper, USDA Agric. Research Service (ARS), Corvallis, OR	NB
<u>Pseudomonas cepacia</u> Burkholder		T. Highley, Forest Products Laboratory (FPL), Madison, WI.	Kings B Media [KB] (2% proteose peptone, 1% glycerol, 0.15% Potassium Phosphate Mono-basic, 0.15% Magnesium sulfate)
<u>P. fluorescens</u> Migula	Pf-5 (#3832)	J. Loper, ARS, Corvallis, OR.	KB
<u>P. putida</u> Migula	A-12	J. Loper, ARS, Corvallis, OR	KB
<u>P. putida</u> Migula	N-1R	J. Loper, ARS, Corvallis, OR	KB
<u>Streptomyces tendae</u>	695 A	Forintek, Ottawa, Canada	Potato-Dextrose-Broth [PDB]
<u>Streptomyces</u> sp.	C 248	Forintek, Ottawa, Canada	PDB
<u>Streptomyces</u> sp.	C 254	Forintek, Ottawa, Canada	PDB
FUNGI			
<u>Gliocladium aurifilum</u>	675 A	Forintek, Ottawa, Canada	Malt agar [MA] (1.5% malt extract, 1% agar)
<u>Gliocladium virens</u> J.H. Miller, J.E. Giddens, & A.A. Foster	H 3	J. Loper, ARS, Corvallis, OR	MA
<u>Penicillium thomii</u> Maire	655 A	Forintek, Ottawa, Canada	MA
<u>Saccharomyces cerevisiae</u> Meyen ex Hansen	591 A	Forintek, Ottawa, Canada	MA
<u>Trichoderma harzianum</u> Rifai	25	Forest Research Lab (FRL, Oregon State University, Corvallis, OR)	MA
<u>T. harzianum</u> Rifai	142	FRL Corvallis, OR	MA

Table 2.2.: Mold and stain fungi employed to evaluate efficacy of bioprotectants.

SPECIES	STRAIN NUMBER
<u>Alternaria</u> <u>alternata</u> (FR:FR) Keissl.	ED 113
<u>Aspergillus</u> <u>niger</u> van Tieghe	BOB
<u>Aureobasidium</u> <u>pullulans</u> [de Bary] G.Arnaud	Zm 3
<u>Bispora</u> <u>betulina</u> (Corda) S.J. Hughes	1
<u>Cladosporium</u> <u>elatum</u> (C. Harz) Nannf.	563-1-1
<u>Hormoconis</u> <u>resinae</u> (Lindau) Arx & G.A. De Vries.	P 1600
<u>Penicillium</u> <u>frequentans</u> Westling	43
<u>Phialophora</u> <u>fastigata</u> (Lagerberg & Melin) Conant	14
<u>Rhinochadiella</u> <u>atrovirens</u> Nannf. in Melin & Nannf.	68
<u>Ulocladium</u> <u>chartarum</u> (G. Preuss) E. Simmon	100

Chemical Company [St. Louis, MO].

2.2.2. Wood Employed

Freshly sawn 5 x 10 cm Ponderosa pine sapwood boards [Pinus ponderosa Laws] were donated by Roseburg Forest Products Inc., Roseberg, Oregon. Boards were immediately cut into 0.5 x 1 x 3.0 cm [tangential x radial x longitudinal direction] test specimens containing only sapwood and stored in a freezer [0°C] until usage.

2.2.3. Preparation of Bioprotectant Inoculum and Suspension

A small amount of bacterial inoculum was taken from a stock culture tube and transferred into a 250 ml Erlenmeyer flask containing 50 ml sterile [121°C, 20 min] liquid growth media appropriate for each organism [Tab. 2.1.]. Inoculated flasks were mounted on a rotary shaker [Lab Line Instr. Inc., Melrose Park, IL] [80 RPM] at 23°C for approximately 65 hours. Cultures were then aseptically harvested by draining the contents of one flask into a 400 ml aluminum tray and adding 200 ml of distilled water to produce a 250 ml bioprotectant suspension. Fungal bioprotectants [except Saccharomyces cerevisiae] were grown on MA plates and incubated at 27°C until abundant sporulation had developed.

Fungal bioprotectant suspensions were then prepared by adding 4 ml of distilled water to each dish to flood spores and hyphal fragments from the surface. The harvested biomass was placed in an aluminum tray [400 ml] and distilled water was added to produce 250 ml of suspension.

2.2.4. Preparation of Stain Fungi Inocula

Fungal stain inocula were prepared by transferring a mycelial plug [3 x 3 mm] from a stock culture tube into a 250 ml Erlenmeyer flask containing 50 ml of sterile [121°C, 20 min] 1 % malt extract media. Cultures were incubated on a rotary shaker [80 RPM] at 23°C and were harvested after 14 days when abundant dark pigmented mycelial biomass had developed. The contents of each flask were vacuum-filtered (1 bar) through Whatman # 4 filter paper using a Buchner funnel. Spores and hyphal fragments were washed off the filter paper into a 500 ml beaker using distilled water. Harvested biomass was then blended for about 10 seconds. Distilled water was added to the blended biomass to obtain 1 L of fungal stain suspension. The fungal stain suspension was drained into a squeeze bottle and kept at room temperature until needed. Fungal stain spores and hyphal fragments produced as described were periodically [approximately in two week intervals] added to the suspension to prevent loss of fungal viability.

2.2.5. Treatment with Bioprotectants and Stain Fungi

Steam sterilized (100°C, 15 min) Ponderosa pine specimens were placed in an aluminum tray [400 ml] and covered with weighted wire to keep wood samples in the suspension during treatment. Two hundred fifty ml of bioprotectant suspension or distilled water [control] were added to the tray for about 8 minutes. The container was then drained and the specimens were removed. The wood samples were allowed to surface dry for approximate two to three minutes before being sprayed to the point of run off on both surfaces with the prepared fungal stain suspension.

2.2.6. Incubation of the Treated Wood Specimens

Wood samples were incubated employing a previously described method [Seifert et al., 1987]. Five sheets of Whatman # 4 filter paper [9 cm in diameter] and a U-shaped glass rod were placed in a 10 cm diameter glass petri dish and autoclaved [121°C, 45 min]. Four wood samples receiving bioprotectant and fungal stain inoculum and one control sample treated with stain fungi only were placed on a glass rod and 5 ml of distilled water was added. Five replicates were established for each bioprotectant evaluated. In addition, dishes receiving only stain fungi or bioprotectant were evaluated for each of the bioprotectants tested. Dishes

were independently coded to minimize any evaluation bias and were then incubated in the dark [27°C] for four weeks. Incubated wood samples were evaluated weekly and deionized water was added to maintain a moist environment.

2.2.7. Evaluations of the Bioprotectants Tested

A modified grading system proposed by Benko (1988) was employed to visually quantify the degree of antagonistic activity against fungal stain. Average degree of stain [ADS] and percentage of fungal stain inhibition [FSI] were determined and calculated, respectively, for each of the bioprotectants evaluated after a four week time period.

Modified grading system used:

0 = unstained wood

[no visible signs of stain]

1 = Incipient establishment of microbial growth

[evidence of microbial growth on surface]

2 = Moderately stained

[at least 1/4 of the wood surface stained]

3 = Heavily stained

[more than 1/2 of the wood surface stained]

2.3. Results and Discussion

Bacterial and fungal bioprotectants differed significantly in their ability to prevent microbial discoloration on unseasoned Ponderosa pine sapwood. The bioprotectants tested can be divided, based on the average degree of stain [ADS] and percentage of fungal stain inhibition [FSI], into three groups. Among the fifteen biological agents investigated, three bioprotectants reduced ADS by more than 25 % when compared with control specimens, while two promoted fungal stain development [ADS > 25%] [Tab. 2.3]. The remaining ten isolates, however, displayed only minor stain inhibition or promotion [Fig. 2.1. a+b].

Bacillus subtilis isolate 733 A completely prevented fungal discoloration on Ponderosa pine sapwood over a four week period [Fig.2.2. a]. Fungal stain was inhibited by approximately 89% in comparison with control samples which already displayed severe [ADS > 3] stain patterns two weeks after inoculation. However, a few wood samples treated with B. subtilis # 733 A developed tiny [1 mm in diameter] zones of microbial growth. In most instances, these spores were visually identified as members of the common airborne genera Aspergillus spp. and Penicillium spp. Evaluation of B. subtilis # 733 A colonized wood was then extended for

Table 2.3.: Average degree of surface stain [ADS] and percentage of fungal stain inhibition [FSI] of bioprotectants evaluated after four weeks after application of selected stain fungi.

SPECIES	ADS [Treated]	ADS [Control]	FSI ^a
<i>B. subtilis</i> 733 A	0.30 ^b [0.46] ^c	2.70 ^d [0.69]	88.88
<i>B. subtilis</i> Q 4000 HB	2.60 [0.74]	2.60 [0.80]	+1.92 ^e
<i>P. cepacia</i>	2.70 [0.64]	2.80 [0.60]	+3.57
<i>P. fluorescens</i> Pf-5	3.00 [0.00]	2.40 [0.92]	+25.00
<i>P. putida</i> A-12	1.70 [0.84]	2.60 [0.80]	34.62
<i>P. putida</i> N-1R	2.70 [0.64]	2.80 [0.60]	+3.57
<i>St. tendae</i> 695 A	2.30 [0.95]	2.80 [0.60]	17.82
<i>Strept.</i> sp. C 248	3.00 [0.00]	3.00 [0.00]	0.00
<i>Strept.</i> sp. C 254	2.75 [0.62]	3.00 [0.00]	8.33
<i>G. aurifilium</i> 675 A	2.75 [0.62]	2.90 [0.30]	5.17
<i>G. virens</i> H 3	1.00 [0.77]	2.77 [0.64]	62.96
<i>P. thomii</i> 655 A	1.80 [0.92]	1.40 [0.80]	+28.57
<i>S. cerevisiae</i> 591 A	2.15 [0.85]	2.30 [0.64]	6.52
<i>T. harzianum</i> 25	2.45 [0.86]	2.80 [0.60]	12.50
<i>T. harzianum</i> 142	2.70 [0.64]	2.40 [0.91]	+12.50

a = $[1 - \text{ADS}/\text{ADSC} * 100 \%$] equation used for computing FSI

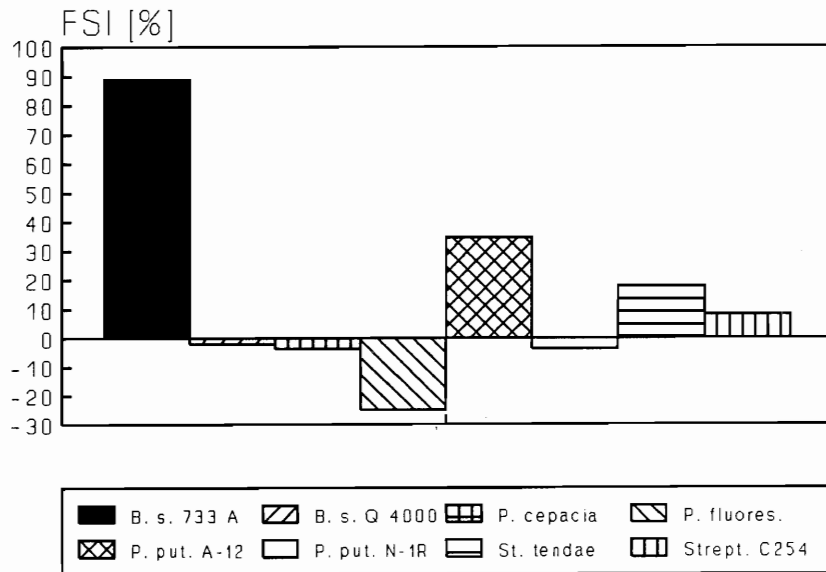
b = value represents mean of 20 replicates

c = values in parenthesis represents standard deviation

d = value represents mean of 10 replicates

e = FSI values indicated with + reflect an increase in staining in comparison with controls

a)



b)

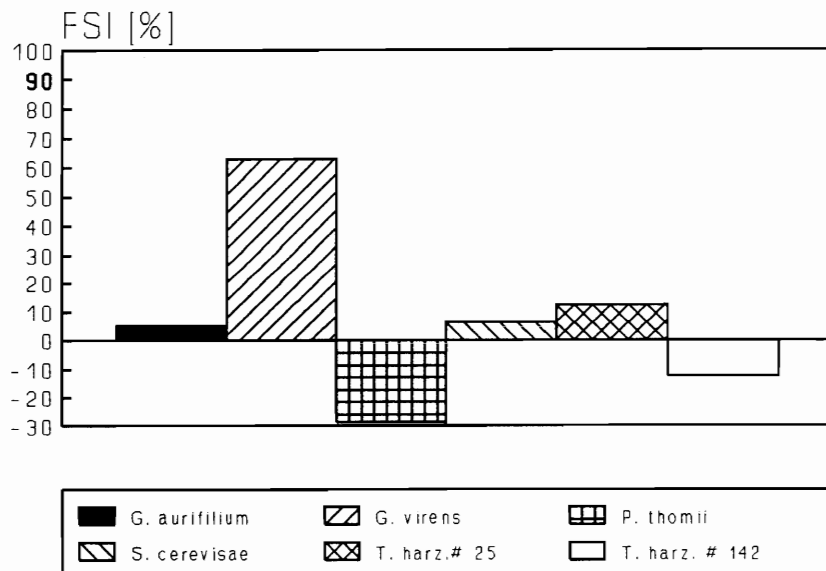


Fig. 2.1.: Percentage of fungal stain inhibition 4 weeks after treatment with potential a) bacterial bioprotectants or b) fungal bioprotectants.

an additional four week period because of the promising results [Fig. 2.2.b]. A gradual decrease in antagonistic activity against mold and stain fungi was observed after 6 weeks. However, no incidence of mold or stain fungi was recorded on 30 % of the wood specimens evaluated, 45 % exhibited only individual moldy spots and 25 % displayed severe [> 3] staining 8 weeks after inoculation. These results suggest that B. subtilis # 733 A delayed fungal invasion, but could not completely prevent growth of mold and stain fungi on unseasoned Ponderosa pine sapwood. Incomplete substrate colonization may have caused the decreasing antagonistic behavior of the biological agent. Thus, wood specimens may still have a sufficient supply of readily available nutrients to support fungal invasion despite bacterial growth.

Previous studies have also suggested that poor colonization affected the level of bioprotection achieved with a different isolate of B. subtilis # 186 [Seifert et al., 1987]. In that study, the author microscopically examined colonization patterns of the bioprotectant in the center of a precolonized pine wood sample and noted that only 20 % of the rays were successfully occupied. Unfortunately, no microscopic investigations were undertaken at this stage of the current study to quantify the ability of B. subtilis # 733 A to colonize the substrate. However, in contrast to earlier studies, no precolonization time was

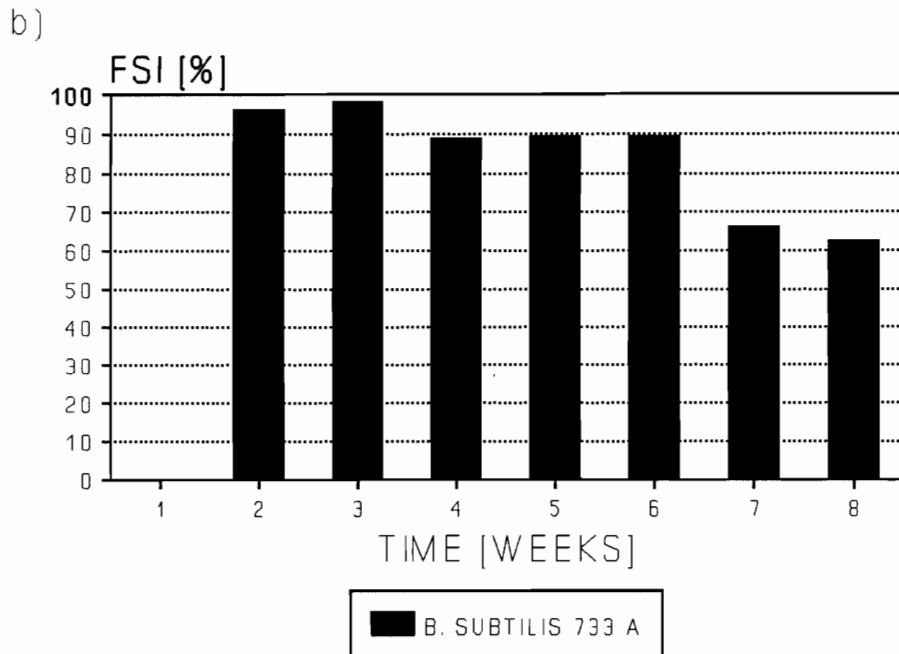


Fig. 2.2.: Bioprotection associated with application of *B. subtilis* 733 A as a) average degree of surface stain [ADS] and b) percentage of fungal stain inhibition [FSI].

provided for the bioprotectant, limiting the potential of the bacterium to completely colonize the wood to inhibit molds and stain fungi.

Competition for nutrients and space has also been suggested as the probable mode of action of B. subtilis [Bernier et al., 1986], but these conclusions were either drawn from dual culture platings on artificial media or from tests of wood soaked [24 hours] in the bacterial culture and then placed over agar media. Thus, the relevance of previous conclusions to the more spartan conditions employed in the present study are questionable since conditions prevalent in wood systems are known to differ to significantly from those in cultures.

Recently, an isolate of B. subtilis was reported to prevent fungal stain growth on rubberwood for a two week period [Florence ,1990]. This wood species is extremely susceptible to fungal stain. The wood samples in this study were dipped for only five seconds in a suspension of biological agent, suggesting that the bioprotectant created a surface barrier or occupied the substrate sufficiently to deny entry by stain fungi. This activity may, however, also reflect the presence of anti-fungal compounds from the biological agent in the applied culture fluid. Longer incubation periods to assess microbial activity would be useful in such instances.

In addition to variations in methods employed,

differences in microbial strains may also explain the differences between results obtained herein and previous investigations. For example B. subtilis isolate Q 4000 HB was also evaluated as a potential bioprotectant in this study and had no effect on the growth of the mold and stain fungi tested. Similar strain variations were reported by Klingström with Scytalidium spp. [1973].

Gliocladium virens # H 3 also exhibited some potential for fungal stain prevention, inhibiting growth of stain fungi by 62 %. This fungus rapidly colonized and occupied the wood substrate as evidenced by the presence of aerial mycelium and green spores 3 weeks after inoculation. In a few instances, molds, which were mostly represented by members of the genus Aspergillus, and stain fungi became established after four weeks of exposure. Towards the end of evaluation periods, mycelium and spores of G. virens were also detected on some control samples which only received fungal stain inoculum, illustrating the ability of the bioprotectant to colonize adjacent substrates. Although no mechanistic studies were undertaken, G. virens is a well known mycoparasite which has been reported to parasitize S. sclerotium and Rhizoctonia solani on agar plates [Tu, 1980]. G. virens has also demonstrated antagonistic action against various pythopathogens [Aluko and Hering, 1970; Howell, 1982; Howell and Stipanovic, 1983]. The G. virens isolate evaluated in this study was able to inhibit fungal stain,

but extensive formation of greenish aerial mycelium and spores was detrimental to the wood appearance. Similar conclusions were drawn by Seifert et al. [1988] in evaluating G. roseum as a potential anti-stain agent. Although growth of the bioprotectant might be limited to the wood surface where it could be brushed off, lysis of bioprotectant spores or mycelium may also stain the wood surface. Unless antifungal compounds produced by strains of G. virens display a special activity against mold and stain fungi prevalent on unseasoned sapwood, introduction of spores or mycelium of this species does not currently represent a useful strategy to protect unseasoned lumber from microbial stain.

Pseudomonas putida isolate A-12 exhibited some potential for bioprotection of unseasoned lumber. While discoloration of wood samples was reduced by approximately 35 % in comparison with control specimens after two weeks, fungal stain did not progress further on bacterially treated specimens. These observations suggest the occurrence of combative interactions such as competition or antibiosis. Antibiotic production has been suggested as a probable mechanism of one P. fluorescens strain inhibiting plant pathogens in culture [Howell and Stipenovic, 1979; Janisiewicz and Roitmann, 1988]. Recently, an antibiotic [phenazine-1-carboxylic-acid] was recovered in situ from the root and rhizosphere soil of wheat grown from seeds

inoculated with a phenazine producing Pseudomonas spp. [Thomashow et al., 1990].

In addition to antibiotic production, bacteria may exert less obvious effects on microbial growth. For example iron competition has also been demonstrated as probable mode of action against phytopathogens. Most microorganisms respond to low iron stress by producing extracellular, low molecular-weight Fe(+3) transport agents which are termed siderophores [Leong, 1986]. However, some fluorescent Pseudomonas strains have shown specific and high affinity for ferric iron and are able to effectively sequester Fe(+3). P. putida # A-12 has been shown to produce siderophores in cultures [Kings B media] as indicated by the presence of a yellow, green, fluorescent pigment, but it is unknown whether in situ production of siderophores occurs. Siderophores play a particularly important role in control of phytopathogens in culture [Loper, 1988], but no evidence for in situ production of siderophores has been found [Loper and Lindow, 1987]. Production of siderophores by a fluorescent Pseudomonas strain was reported to reduce chlamydospore germination in the rizosphere of plants [Elad and Baker, 1985].

Although application of T. harzianum # 25 as a potential biological agent was detrimental to the wood appearance because of a yellowish stain produced by the bioprotectant, some interesting observations were made. The

yellow stain and conspicuous green spores were first observed two weeks after application when most control samples already exhibited progressive [stain ratings > 3] stain patterns. Growth of mold or stain fungi was not observed on yellow stained wood specimens. These observations may suggest the production of antimicrobial secondary metabolites in or associated with the yellow pigment.

In vitro production of volatile as well as non-volatile secondary metabolites [antibiotics] by Trichoderma spp. which inhibited selective decay fungi has been reported [Dennis and Webster, 1971 a+b; Bruce et al., 1984, 1987; Highley and Ricard, 1988]. Furthermore, cores removed from distribution poles treated with a biological agent, Binab FYT, containing two Trichoderma spp. only resisted decay when Trichoderma spp. had successfully occupied the substrate. Although decay bioprotection was not an objective in the current study, observations described may justify additional research to quantify decay susceptibility of the wood samples colonized by T. harzianum # 25.

It is further interesting to note that the yellow stain and green spores were also recorded on some control samples which suggests combative interactions are occurring to capture an already occupied resource. Thus, production of volatile compounds, which have been reported to be produced by Trichoderma spp. [Dennis and Webster, 1971 b; Bruce et

al., 1984, 1987] may also have assisted bioprotectant colonization of the substrate already discolored by mold and stain fungi. Secondary resource capture may have further been accomplished through a combination of parasitic actions and the production of antibiotic compounds, both of which have been demonstrated with T. harzianum in culture [Dennis and Webster, 1971 c; Murmanis et al., 1988] but not on wood.

Trichoderma harzianum # 142, was associated with slight increases in staining [FSI = 12.5 %] on wood specimens and no evidence of inhibition as noted for T. harzianum # 25 were found.

Generally, T. harzianum # 142 was apparently unable to completely colonize the substrate when competing with molds and stain fungi. Only in a few instances were green spores of this bioprotectant present on the wood surface of bioprotectant/stain fungus treated samples 2 weeks after inoculation, but all specimens treated only with the biological agent [control dish] contained green spores at the same time. Green spores produced by isolates of T. harzianum generally indicate substrate colonization [Bruce and Highley, 1991]. Thus, the results obtained with T. harzianum # 142 suggest that this biological agent was unable to compete with microflora on the substrate. The results also underscore the importance of understanding strain variations of a potential bioprotectant species.

Penicillium thomii # 655 A received a relatively low

ADS [1.8] after 4 weeks of evaluation, but staining was not inhibited [FSI = + 28.57] when compared with control samples. Control samples, however, achieved the lowest ADS of the controls. The low degree of staining of controls may reflect natural variations within the wood species employed. However, the surface of wood specimens inoculated only with P. thomii # 655 A were covered with a dry mass of grey-green spores after a four week period making this treatment unacceptable for bioprotection against fungal stain. Seifert et al. [1988] noted that an isolate of P. thomii tended to form sclerotia on the wood surface and thus impaired the wood appearance, although P. thomii # 665 B was identified as a promising bioprotectant for preventing microbial discolorations.

Pseudomonas cepacia was unable to protect wood specimens against microbial staining and thus did not inhibit staining. In previous tests, Benko [1988] identified P. cepacia # 6253 as a promising biological agent on agar media. Subsequent trials on Pinus radiata using a cell free culture broth of the same isolate produced complete stain protection for 8 weeks under laboratory conditions [Benko, 1989]. The different and disappointing outcome achieved herein with P. cepacia may, again, highlight strain variations. Furthermore, the different media used in Benko's study may have promoted the production of antimicrobial compounds not present in the King's B media

employed in this study. Our results suggest that P. cepacia was unable to compete with fungal competitors for nutrients and space.

Generally, failures of the bioprotectants evaluated to prevent microbial discolorations on Ponderosa pine specimens may reflect inappropriate media or culture conditions. The inability to compete with microbial competitors once introduced on the substrate or the characteristics of the wood may also have limited bioprotectant performance. For example, potential biological agents prevalent in the soil environment may be unable to utilize nutrients present on unseasoned sapwood of Ponderosa pine. These variations in physiologic capabilities highlight the need for more detailed studies of the effects of microenvironment on performance of bioprotectants.

2.3.1. Conclusions

B. subtilis # 733 A was identified as a promising bioprotectant which completely prevented microbial discoloration on Ponderosa pine sapwood. After 6 weeks, however, a gradual decrease in protection was noted.

The wood assay used in this study for evaluation of potential bioprotectants, although different when compared with natural conditions, should produce more realistic results than agar plate tests. However, specimen size should

be larger to increase the accuracy of microbial stain assessments. Additionally, the grading system employed in these studies did not allow quantification of intensity of wood discolorations and, furthermore, relied on subjective observations. Variations in wood appearance within Ponderosa pine specimens also interfered with the grading system employed. The development of more objective stain assessment systems which eliminate human bias would significantly improve the accuracy of stain tests. The use of optical scanning may represent one such method for improving stain assessment.

3.0. Evaluate the effect of media on the effectiveness of selected bioprotectants

3.1. Literature Review - The use of Bacillus subtilis in biotechnology

Members of the genus Bacillus are aerobic, endospore-forming, gram positive bacteria. This genus represents one of the most diverse and economically useful groups of microorganisms. Bacillus spp. are widely distributed in air, soil, decomposing organic matter and aquatic systems [Harwood, 1889].

The metabolic diversity of Bacillus spp. has led to their utilization for various industrial processes. For instance, soybeans are fermented into Natto using a B. subtilis strain. Japan consumes 6×10^6 kg of B. subtilis produced Natto annually [Harwood, 1989]. Bacillus spp. are used for production of antibiotics, enzymes and insecticides. Bacillus thuringiensis, a cosmopolitan soil colonizer [Martin and Travens, 1989], represents one of the most successful biological control agents [Priest, 1990; Seifert, 1990]. This bacterium produces a toxic crystalline protein which is ingested by the insect larvae feeding on leaves.

Bacillus subtilis has been extensively studied and our

understanding of this species is second only to E. coli [Harwood, 1989]. Strains of B. subtilis are considered to be non-pathogenic and the Food and Drug Administration considers them to be "generally safe" [Harwood, 1989]. As a result, this species is widely employed for control of plant pathogens and post harvest diseases.

Antifungal compounds isolated from culture fluids of B. subtilis have been reported to inhibit a wide range of Ascomycetes and Fungi Imperfecti [Walton and Woodruff, 1949; Babad et al. 1952; Kugler et al., 1990]. Antifungal substances produced in liquid cultures of B. subtilis have also been suggested as the probable mechanism by which plant pathogens are inhibited when exposed to suspension and cell free culture broths [Dunleavy, 1955; Swinburne et al. 1975; Baker et al., 1983; Utkede and Rahe, 1983; Pusey and Wilson, 1984; Baker and Stavely, 1985; McKeen et al. 1986; Rytter et al., 1989; Ferreira et al. 1991]. Application of one B. subtilis isolate has also been reported to increase crop yield although the target pathogen was not effectively controlled [Merriman et al.; 1974;].

Isolates of B. subtilis have also been employed for bioprotection of unseasoned sapwood from microbial discoloration [Bernier et al., 1986; Florence et al., 1990; Seifert et al., 1987]. In these studies production of antibiotics and competition for nutrients and space were suggested as probable modes of action.

In a recent study, media composition has been reported to significantly change performance of bioprotectants evaluated for protection against wood staining microorganisms [Benko and Highley, 1990 a+b]. Rytter et al. [1989] reported that Eugon broth used as the culture medium enhanced the antagonistic effects of B. subtilis against Puccinia pelargonii-zonalis Doidge, the causal agent of geranium rust, when compared with cultures grown in nutrient broth. Media composition undoubtedly affects biosynthesis of secondary metabolites. Small variations in media composition or culture conditions will change quality and quantity of antifungal compounds produced in a culture broth [Ozcengiz et al., 1990].

The development of effective bioprotectants will require consistent performance. The conditions under which the organism is grown prior to application will have substantial influences on the fitness of these isolates. Identifying media which optimize microbial growth or production of secondary metabolites may therefore represent a fruitful avenue for improving the prospects for successful bioprotection.

3.2. Material and Methods

3.2.1. Bioprotectants and Media Evaluated

Bacillus subtilis # 733 A, identified as a promising bioprotectant against microbial sapwood discolorations in the initial screening trial and B. subtilis isolate ATCC 6633 were employed to quantify the effects of media composition on bioprotectant capability against staining fungi. The latter isolate has previously been employed to produce antibiotics [Kugler et al., 1990]. Media as well as culture conditions were selected from previous studies [Tab. 3.1.]. Media chemicals were purchased from Difco Laboratories [Detroit, MI], or Sigma Chemical Company [St. Louis, MO].

3.2.2. Preparation of Bioprotectant Inoculum

A small plug of bacterial inoculum was taken from a nutrient agar culture tube and transferred into a 2.8 l flask holding 1.5 l of sterile media [121°C], 20 min] [Tab. 3.1]. When "0.3 NB" media was tested, the bioprotectants were cultured in 250 ml Erlenmeyer flasks containing 50 ml of sterile nutrient broth. The contents of six flasks [300 ml] were added to 1.2 l of distilled water to prepare 1.5 l of this growth media. Bioprotectant cultures were then

Tab. 3.1.: Media and culture conditions employed to evaluate the effects of nutrient levels on the performance of bioprotectants against wood staining fungi.

MEDIA	CULTURE CONDITIONS			REFERENCE
	Time [hrs]	Temp[°C]	RPM	
Nutrient broth [NB]	60	25	80	Claus & Fritz, 1989
PA [PA]	18	37	225	Ozcengiz <u>et al.</u> , 1990
Penassay broth [PB]	48	30	250	Bernier <u>et al.</u> , 1986
PL [PL]	48	25	120	Kugler <u>et al.</u> , 1990
Tryptic Soy-broth [TSB]	72	37	250	Anker <u>et al.</u> , 1948
Yeast-dextrose-carbonate [YDC]	72	25	80	Wilson <u>et al.</u> , 1967
Yeast-malt-dextrose broth [YMDB]	25	25	80	Seifert <u>et al.</u> , 1987

incubated according to conditions listed [Tab. 3.1].

3.2.3. Treatment of Wood with Bioprotectant Culture Fluids and Fungal Stain Inocula

Treatment procedures described in the initial screening trial were employed with slight modifications. The harvested culture fluid was drained into an aluminum container [19.5 x 23.4 x 6.3 cm] and 120 steam sterilized [121°C, 15 min] Ponderosa pine specimens [0.5 x 1.5 x 3.0 cm] were dipped for 8 min each into the bioprotectant suspension. Control samples were only immersed in distilled water [300 ml]. The dipped specimens were removed, placed on a tray, allowed to surface dry and abundantly sprayed with the suspension of stain fungi [Tab. 3.2.]. The specimens were distributed into glass petri dishes and incubated in the dark at 20, 26, or 32 °C. Incubated specimens were examined weekly for evidence of microbial stain and 5 ml of distilled water was periodically added to maintain a moist environment. In addition, one half of the treated wood samples were resprayed with fungal stain inoculum at two week intervals to simulate repeated influxes of staining organisms.

Table 3.2.: Mold and stain fungi employed to evaluate bioprotectant capability on Ponderosa pine sapwood.

SPECIES	STRAIN Number
<u>Alternaria alternata</u> (FR:FR) Keissl.	ED 113
<u>Aspergillus niger</u> van Tieghe	BOB
<u>Aureobasidium pullulans</u> [de Bary] G.Arnaud	Zm 3
<u>Bispora betulina</u> (Corda) S.J. Hughes	1
<u>Ceratocystis pilifera</u> (Fries) C. Moreau	55 A
<u>Cladosporium elatum</u> (C. Harz) Nannf.	563-I-1
<u>Hormoconis resinae</u> (Lindau) Arx & G.A. De Vries.	P 1600
<u>Penicillium frequentans</u> Westling	43
<u>Phialophora fastigata</u> (Lagerberg & Melin) Conant	14
<u>Rhinoctadiella atrovirens</u> Nannf. in Melin & Nannf.	68
<u>Ulocladium chartarum</u> (G. Preuss) E. Simmon	100

3.2.4. Evaluation of Media Effects on Bioprotection

The effects of media on the performance of the biological agents tested were assayed 8 weeks after inoculation. Surface stain [ADS] and percentage of stain inhibition [FSI] were determined and computed as previously described. Wood specimens were then longitudinally split and the percentage of internal fungal stain inhibition [IFSI] was determined based on the following grading system:

- 0 = unstained wood
[no visible signs of internal stain]
- 1.0 = small [1 or 2 mm in diameter] stained spots visible near the end grain
- 1.5 = a thin [0.5 mm] stained shell enclosing no more than one-fourth of the circumference
- 2.0 = stained shell [0.5 mm] encircling no more than one-half of the circumference
- 2.5 = stained shell [0.5 mm] enclosing more than one-half of the circumference
- 3.0 = stained shell [0.5 mm] enclosing the wood sample
- 4.0 = more than 50 % of the internal wood surface area stained

3.3. Results and Discussion

Incubation of bioprotectants on 8 different media prior to wood application dramatically altered the degree of stain prevention. Media preferences were noted with the biological agents employed and these preferences positively affected bioprotectant performance. For example, Bacillus subtilis # 733 A grown on "0.3 NB" produced the best bioprotection against microbial stain at all temperature levels tested, while Penassay broth supported the bioprotectant B. subtilis ATCC 6633 at most temperatures tested [Tab. 3.3.; 3.4.]. However, percentage of fungal stain inhibition [FSI] and percentage of internal fungal stain inhibition [IFSI] achieved by the two biological agents using their preferential media suggested that B. subtilis # 733 A provided superior bioprotection to unseasoned Ponderosa pine sapwood. In fact, the FSI achieved at 26 °C [not resprayed], the conditions which were employed in the initial screening trial of this study, confirmed the outcome produce by B. subtilis # 733 A from the first screening. Although 1.5 l of bacterial suspension was used to treat Ponderosa pine samples, the ratio [1:4, culture fluid to distilled water] of the composed bioprotectant suspension and culture conditions [23 °C, 80 RPM] remained constant.

Antibiotic synthesis by Bacillus spp. generally occurs in the early stages of sporulation and as the culture enters

Tab. 3.3.: Percentage of surface and internal stain inhibition [%] ^a achieved with <i>B. subtilis</i> # 733 A after 8 weeks of evaluation.						
	Temperature [°C]					
	32		26		20	
MEDIA	Sprayed ^b	Not ^c	Sprayed	Not	Sprayed	Not
1.5 NB	+5.50 [+87.69] ^d	+31.81 [+22.58]	13.33 [11.42]	11.66 [68.18]	5.0 [47.54]	16.66 [63.63]
0.3 NB	0.00 [5.60]	27.58 [54.74]	38.88 [43.0]	55.76 [86.84]	+14.58 [28.00]	14.00 [96.66]
PB	3.66 [+83.07]	4.16 [+150.0]	16.66 N.D. ^e	1.66 N.D.	8.33 [96.06]	6.60 [N.D.]
YMDB	+9.25 [+167.0]	+46.66 [+530.0]	0.00 [5.41]	0.00 [9.37]	1.66 [9.18]	1.66 [8.33]
YDC	0.00 [+203.0]	0.00 [+94.73]	0.00 [3.20]	0.00 [3.92]	0.00 [34.78]	0.00 [10.98]
TSB	0.00 [+940.0]	0.00 [+500.0]	0.00 [+9.77]	0.00 [+40.0]	0.00 [+21.00]	0.00 [+20.0]
PA	10.00 [40.00]	12.50 [44.61]	0.00 [17.31]	0.00 [20.60]	0.00 [9.23]	0.00 [9.70]
PL	+22.91 [+71.42]	+11.11 [+460.0]	1.66 [13.46]	0.00 [+11.11]	0.00 [32.00]	0.00 [32.00]

a = $[1 - (\text{ADS}/\text{ADCS}) * 100 \%$ equation used for computing percentage of surface-, and internal stain inhibition.

b = samples were independently sprayed three times with a fungal stain suspension.

c = samples were not independently sprayed three times with a fungal stain suspension.

d = values in parenthesis represents percentage of internal stain inhibition.

e = Not determined since wood samples or control specimens exhibit no signs of internal stain.

Tab. 3.4.: Percentage of surface and internal stain inhibition [%] ^a achieved with <i>B. subtilis</i> ATCC 6633 after 8 weeks of evaluation.						
	Temperature [°C]					
	32		26		20	
MEDIA	Sprayed ^b	Not ^c	Sprayed	Not	Sprayed	Not
1.5 NB	5.00 [70.73] ^d	0.00 [20.00]	6.66 [16.84]	0.00 [58.06]	5.00 [59.58]	0.00 [14.76]
0.3 NB	15.00 [90.00]	11.53 [33.33]	3.33 [48.42]	0.00 [21.25]	0.00 [17.14]	0.00 [36.11]
PB	5.00 [75.00]	27.00 [90.00]	16.66 [73.58]	13.30 [97.61]	18.51 [92.41]	1.66 [16.82]
YMDB	+13.88 [N.D.] ^e	30.00 [0.00]	3.33 [24.19]	11.66 [53.48]	0.00 [14.75]	0.00 [25.00]
YDC	+20.00 [+84.21]	+7.14 [+414.0]	0.00 [+27.55]	0.00 [+25.00]	0.00 [+12.54]	0.00 [+13.72]
TSB	+11.11 [+275.0]	+87.50 [+205.0]	0.00 [+15.00]	0.00 [+1.88]	+3.44 [4.72]	0.00 [+4.61]
PA	11.11 [53.63]	10.71 [12.72]	0.00 [6.45]	0.00 [14.72]	0.00 [8.82]	0.00 [4.12]
PL	13.33 [+103.0]	1.66 [+13.33]	0.00 [+8.51]	0.00 [14.09]	0.00 [21.42]	0.00 [23.80]

a = $[1 - (\text{ADS}/\text{ADCS}) * 100 \%$ equation used for computing percentage of surface-, and internal stain inhibition.

b = samples were independently sprayed three times with a fungal stain suspension.

c = samples were not independently sprayed three times with a fungal stain suspension.

d = values in parenthesis represents percentage of internal stain inhibition.

e = Not determined since wood samples or control specimens did exhibit no internal stain.

the stationary phase in the culture medium [Priest, 1989]. It is possible that B. subtilis # 733 A entered the stationary phase in the optimal culture medium [50 ml NB] more rapidly and thus produced antifungal compounds at an earlier time point. Establishing growth curves for B. subtilis # 733 A might have supported this hypothesis, although Bacillus spp. which lack the ability to synthesize antifungal compounds have been reported to display "normal growth characteristics" and thus to produce growth curves similar to antibiotic producing strains of Bacillus spp. [Priest, 1989]. However, the use of a cell free culture broth of "0.3 NB" to treat Ponderosa pine specimens against sapwood staining microorganisms would help to determine if competing fungi were inhibited solely by antifungal compounds present in the treatment fluid.

Although media affected the performance of B. subtilis # 733 A, incubation temperature of treated [bioprotectant/fungal stain] wood specimens also influenced results. Bioprotection achieved with the "0.3 NB" treatment was better at the higher [32°C] incubation temperature levels than lower [20°C] temperature, but PSI values were lower in specimens incubated at both temperatures than in those incubated at 26°C, which produced a 56 % reduction of surface stain [Fig. 3.1. a]. Interestingly, optimum growth of C. pilifera has been reported to occur at 26°C [Käärrik, 1974]. Favorable incubation conditions [temperature]

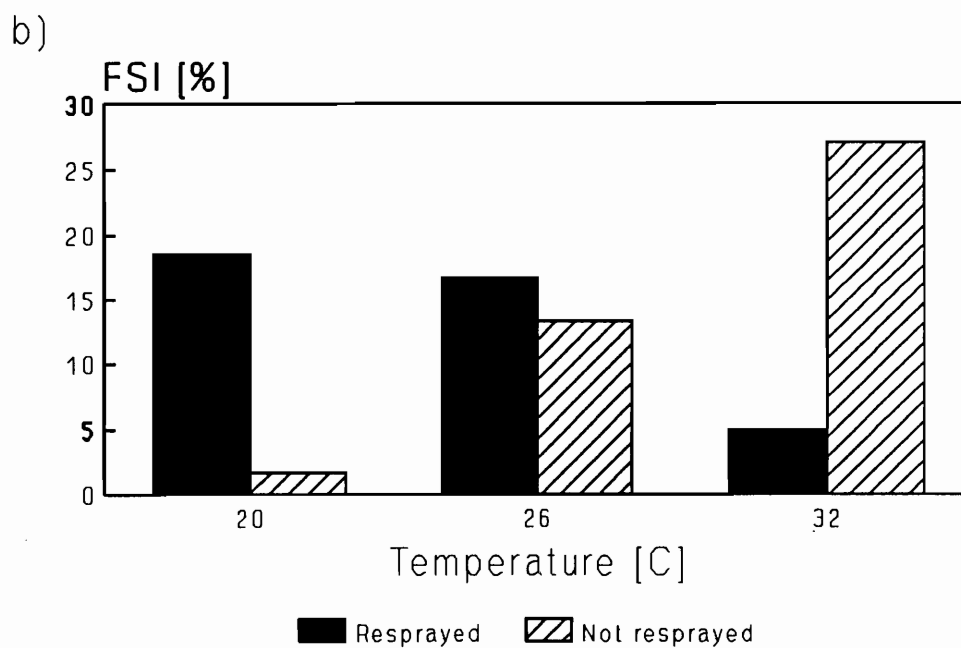
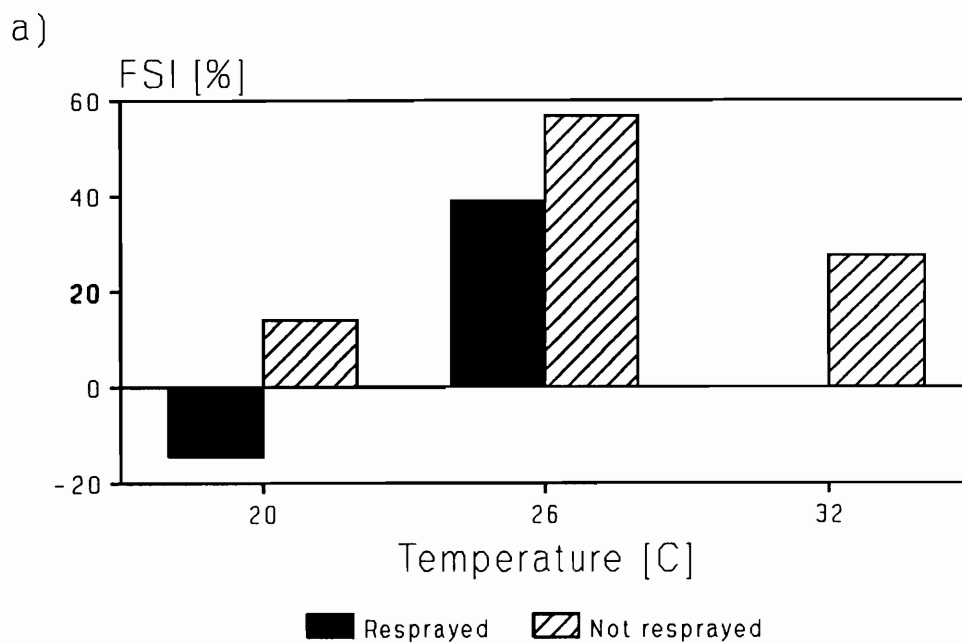


Fig. 3.1.: Effects of preferential media on the ability of bioprotectants to protect Ponderosa pine from microbial stain a) *B. subtilis* 733 A with a "0.3 NB" or b) *B. subtilis* ATCC 6633 with a "PB".

may have enabled the biological agent to rapidly exploit readily available nutrients and, thus, may have excluded microbial competitors. Furthermore, antifungal compounds, possibly produced in the culture broth of the biological agent may have remained active for a longer periods at this temperature.

Inoculum density also influenced performance of B. subtilis # 733. Decreased FSI's and IFSI's were noted when wood specimens were resprayed with fungal stain inocula at 2 week intervals at all temperature levels tested, when compared to values obtained for the non-resprayed treatment. Although inoculum levels present on resprayed samples may be higher than normally found on exposed sapwood, they suggest that successful bioprotection depends to some extent on the level of fungal stain inoculum. Alternatively, the loss of protection with respraying may reflect gradual loss of antibiotics from the wood surface. Resprayed spores could then germinate in the absence of inhibitory compounds. At 26°C, however, B. subtilis # 733 A was still able to reduce surface stain and internal stain by approximately 40 %, suggesting that this temperature range was favorable for continued activity of the bioprotectant against wood staining fungi.

The "1.5 NB" treatment produced only a slight reduction in surface stain at 26°C and 20°C and was associated with increased surface staining at 32°C. Although the "1.5 NB"

treatment contained the same ingredients used for composition of the "0.3 NB" treatment, the increased nutrient levels apparently had more dramatic effects on growth. Furthermore, B. subtilis # 733 A was cultured in a 2.8 l flask, which may have changed culture conditions, such as oxygen supply. These changes may have impaired production of antimicrobial compounds in 1.5 l NB. In addition, increased deposition of nutrients on the wood surface with the more concentrated media may have inadvertently supported fungal stain infestation on the wood.

Growth of B. subtilis # 733 A on PA, PL, TSB, YDC or YMDB media produced inoculum that did not effectively prevent surface stain. Increased IFSI values produced with PL, TSB, YDC and YDMB media indicated that nutrient depositions on the wood surface may have supported rapid fungal stain growth and penetration through the wood substrate.

PA, PL and TSB have been successfully employed to produce antifungal compounds by B. subtilis isolates [Anker et al., 1948; Kugler et al., 1990; Ozcengiz et al., 1990]. Although, culture conditions [temperature, time, RPM] approximated those previously employed, the quantity of media employed [1.5 l] in a 2.8 l flask may have changed culture conditions. As a result antibiotics might have been produced in insufficient quantities to suppress microbial growth on wood.

Bacillus subtilis ATCC 6633 exhibited some potential to reduce both surface and internal microbial stain on Ponderosa pine at all temperatures tested when this species was grown on Penassay broth [PB] [Fig.3.1 b]. This media has been used to evaluate the potential of B. subtilis isolate # 186 to prevent microbial stain [Bernier *et al.*, 1986]. This biological agent has been identified as potential bioprotectant and is believed to function by competition and production of antibiotics. At 32°C incubation temperature, a 27 % reduction of surface stain was noted with specimens which were not resprayed, while 18 % FSI was achieved when resprayed specimens were incubated at 20°C. The "0.3 NB" treatment only showed slight bioprotection at 32°C, while "1.5 NB" treatment produced no reduction of surface stain. Although Bacillus spp. grew well in commercial nutrient agar [Priest, 1989], the NB composition and culture conditions herein employed might be unsuitable for production of antifungal compounds by B. subtilis. Bacterial strains can vary widely in physiologic capabilities, a factor that may explain the variable responses of the two Bacillus isolates in this study. In addition, growth of B. subtilis ATCC 6633 on PA, PL and YDMB also produced slight reductions in surface stain at 32°C, but with the exception of YDMB, which also showed surface stain inhibition at 26°C, sapwood staining microorganisms were not excluded in samples incubated at 20 or 26°C.

"PL" medium has been employed for production of Rhizoctin A, an antifungal compound, using B. subtilis ATCC 6633 [Kugler et al., 1990]. Variations in culture conditions may have prevented production of this compound or may have reduced the quantities produced. Furthermore, Rhizoctin A may have no activity against the wood staining microorganisms evaluated or may have rapidly been deactivated through the activity of proteolytic enzymes on the wood substrate. Adsorption to the substrate may have further reduced potential fungicidal effects.

Media composition has been reported to influence bioprotectant performance against wood staining microorganisms [Benko and Highley, 1990 a + b]. However, previous studies have only evaluated the activity of fungitoxic compounds produced in a bacterial culture fluid against Ceratocystis coerulescens and Trichoderma harzianum and did not include the variety of organisms used herein. Although C. coerulescens and T. harzianum represent two very important sapwood colonizing microorganisms, they might behave differently when competing with other sapwood staining fungi. Furthermore, the performance of antifungal compounds, produced in a culture fluid, in a fluctuating environment remains unclear. For instance, these antimicrobial compounds may leach from the wood or become deactivated. In addition to antifungal compounds, the presence of the bioprotectant on the wood substrate which is

capable of sequestering readily available nutrients from the substrate would substantially improve the prospects for successful bioprotection. However, antifungal compounds are considered to exhibit a key role in suppressing microbial competitors in the early stage of infestation and their presence may provide an initial advantage for the bioprotectant.

Our results indicate that the media employed has a significant effect on bioprotection and must be carefully considered in field trials.

3.3.1. Conclusions

Media composition influences performance of bioprotectants against wood staining microorganisms. B. subtilis 733 A inhibited microbial stain on Ponderosa pine specimens when a "0.3 NB" treatment was applied; however, the differences between the "1.5 NB" and the "0.3 NB" treatment suggest that media composition should be more closely investigated. For instance, altering C:N ratio, micronutrient concentration, incubation temperature or other environmental factors may significantly improve biological activity against microbial stain on wood.

4.0. Evaluate the Fungitoxic Effects of Extracts from Ponderosa Pine Samples Inoculated with a Bioprotectant and/or Sapwood Staining Microorganisms

4.1. Introduction

Biological control research in agriculture has demonstrated that several mechanisms function in protection against phytopathogens, including antibiosis, competition and mycoparasitism [Baker, 1983; Burge, 1988; Campbell, 1989; Schmidt, 1990]. The production of antimicrobial compounds in culture broth of the biological agent has been reported in numerous studies and is thought to play a key role in bioprotection [Gottlieb and Shaw, 1970; Gottlieb, 1976; Fravel, 1988].

Most biocontrol studies have employed defined nutrient media to promote production of antibiotics [Weindling, 1937, 1941; Howell and Stipanovic, 1979, 1982; Dunlop *et al.*, 1989], but natural microhabitats associated with plants are likely to have limited carbon and nitrogen levels which may not support production of antibiotics [Campbell, 1989]. Wood, with its high C:N ratio, provides an even greater challenge to potential antibiotic producing biological control agents. Even if they are produced in wood, antibiotics might be readily inactivated by adsorption to minerals or organic particles in soil [Gottlieb, 1976] or

may be decomposed by proteolytic enzymes, resulting in a loss of antimicrobial activity [Campbell, 1989].

Furthermore, these compounds are most likely produced in very small quantities and thus are very difficult to detect analytically in the natural environment [Thomashow et. al., 1990].

In vivo production of antibiotics has rarely been reported. Recently, Thomashow et al. [1990] demonstrated in situ production of an antibiotic, Phenazine - 1 - Carboxylic Acid, by a fluorescent Pseudomonas spp. in the rhizosphere of wheat.

Secondary metabolites produced in a culture broth of a biological agent have been reported to inhibit growth of wood staining microorganisms on agar media and on sapwood [Croan and Highley, 1991 a+b; Highley et al., 1991], but no attempts have been made to demonstrate in situ production of fungitoxic compounds. In situ production of antimicrobial substances may represent an important mechanism in bioprotection against fungal stain. Understanding these mechanisms may permit further enhancements in bioprotectant performance either through strain selection or by selectively modifying the environment to favor the biological agent.

4.2. Material and Methods

4.2.1. Treatment and Incubation of Ponderosa Pine Specimens

B. subtilis # 733 A was inoculated into 6 Erlenmeyer flasks [250 ml] each containing 50 ml of nutrient broth [NB] and incubated on a rotary shaker [60 hrs, 80 RPM] at room temperature. A bacterial suspension [1.5 l] was prepared by combining the contents of 6 flasks with 1.2 l distilled water. Steam sterilized [100°C, 15 min] Ponderosa pine specimens [0.5 x 1.5 x 3 cm] were then dipped into the bacterial suspension for approximately 8 minutes. Treated samples were then sprayed with a suspension of spores and hyphae of stain fungi [Tab. 4.1.], placed into glass petri dishes and incubated [26°C] according the method previously described. In addition, samples only treated with the bioprotectant, the fungal stain suspension or into distilled water [300 ml] were used as controls.

4.2.2. Extraction of Incubated Wood Samples

Ten treated [bioprotectant and fungal stain suspension] Ponderosa pine specimens were removed from the incubator after a 1, 2, 3 or 4 week period, placed into 2 Erlenmeyer flasks [250 ml] each containing 50 ml of 0.001 % Tween 80

Table 4.1.: Mold and stain fungi employed to evaluate potential fungitoxic compounds produced by *B. subtilis* # 733 A compounds on Ponderosa pine.

SPECIES	STRAIN NUMBER
<i>Alternaria alternata</i> (FR:FR) Keissl.	ED 113
<i>Bispora betulina</i> (Corda) S.J. Hughes	1
<i>Ceratocystis pilifera</i> (Fries) C. Moreau	55 A
<i>Cladosporium elatum</i> (C. Harz) Nannf.	563-I-1
<i>Hormoconis resinæ</i> (Lindau) Arx & G.A. De Vries.	P 1600
<i>Phialophora fastigata</i> (Lagerberg & Melin) Conant	14
<i>Rhinoctadiella atrovirens</i> Nannf. in Melin & Nannf.	68
<i>Ulocladium chartarum</i> (G. Preuss) E. Simmon	100

[Manooleate] and shaken [120 RPM] for 10 hours at room temperature. The extracts were combined and filtered using a sequence [0.8, 0.45, 0.22 μ] of filter membranes. The filtrate was then concentrated in an Amicon Stirred Ultra-Filtration Cell [Amicon Inc., Danvers, MA] using Amicon Diaflo YM 10 and YM 3 membranes [Amicon Inc., Danvers, MA] to produce molecular weight cut offs of < 3,000, 3,000 to 10,000 and > 10,000. The concentrated extracts were then stored at 4°C until used. Control samples were prepared according to the same procedure.

4.2.3. Evaluation of Fungitoxicity of Harvested, Concentrated Extracts against Sapwood Staining Microorganisms

A depression [0.4 cm in diameter, 0.3 cm in depth] was cut into the center of a petri dishes containing malt agar [1.5 % malt extract, 1 % agar] medium. A small [0.05 ml] amount of extract was then pipetted into this depression. Each petri dish was then abundantly seeded with a suspension of spores and hyphae [Tab. 4.1]. Seeded malt agar dishes were then incubated in the dark at 26°C and evidence of zones of inhibition around the well were measured after 7 days of incubation. Each treatment [bioprotectant-stain, bioprotectant, fungal stain, distilled water] was performed in two replicates. In addition, dishes receiving only the

concentrated extract [0.05 ml] or seeded with fungal stain suspension [Tab. 4.1] served as controls within each treatment.

4.3. Results and Discussion

Petri dishes treated with filter sterilized [0.22 u, 47 mm diameter filters] extract concentrated to 3 different molecular weight cut offs and sprayed with a fungal stain suspension [Tab. 4.1] showed no evidence of zones of inhibition at any treatment levels tested. Extracts harvested from incubated wood specimens 1, 2, 3, or 4 weeks after incubation did not inhibit growth of the mold and stain fungi evaluated. In fact, each seeded malt agar petri dish exhibited abundant mycelial growth of wood staining organisms after 7 days of incubation.

This rather disappointing outcome may reflect the extraction procedure used, or the solvent employed. In addition, toxic compounds which might have been produced in situ may have been tightly bond to the wood substrate [Dawson-Andoh and Morrell, in press]. Tween 80 has been used in a recent study to enhance extraction of tightly bound extracellular proteins from wood specimens inoculated with a selected decay fungus [Dawson-Andoh and Morrell, in press]. Antibiotics have been demonstrated to be adsorbed to minerals or organic particles in soil [Gottlieb, 1976] and

might also have been bond to the wood when produced in situ by a biological agent.

Unsuitable solvent conditions, changes in pH in the solvent employed caused by wood leachates [soluble nutrients, natural wood extracts], or the use of a lower solvent concentrations [0.001 %] might have created an unfavorable environment for extraction. Thus, fungitoxic compounds produced in situ might not be released under the employed conditions or may have been inactivated in the process of extraction.

B. subtilis # 733 A might also have produced volatile compounds which diffuse and detrimentally affect competing sapwood staining microorganisms. For instance, some Trichoderma spp. have been reported to produce volatile compounds on nutrient media and to inhibit selected wood decay fungi [Dennis and Webster, 1971 b; Bruce et al., 1984, 1987]. Volatile compounds produced by an biological agent are advantageous because direct physical contact between competing populations would not be needed for exclusion of competing microorganisms.

It is also possible that neither volatile compounds nor antifungal substances were produced in situ. Production of antibiotics is constrained by various biological and physical factors [Fravel, 1988; Campbell, 1989], but nutritional limitations are believed to be of most importance in bioprotection [Thomashow et al., 1990]. Thus,

nutrients present in wood may not support production of antifungal compounds [Faull, 1988]. Wood is considered to offer only limited amounts of nutrients and these levels may have been insufficient for in situ production of antibiotics by the biological agent tested.

Fungitoxic compounds produced in liquid cultures and applied to unseasoned sapwood in either a suspension or in a cell free supernatant have been reported to inhibit fungal stain development under laboratory conditions [Croan and Highley, 1991 a+b; Highley et al., 1991]. Although this approach resembles conventional [chemical] anti-stain treatment, the production of these compounds under suboptimal field conditions remains to be demonstrated.

Our results suggest that competition for nutrients and space represent the dominant modes of action in this bioprotection system. Culture fluids containing antimicrobial compounds may assist suppression of competitors in the initial stage, however, the possibility that the activity of these compounds decreases with time, particularly in a fluctuating environment, highlights the need for microorganisms which successfully compete for nutrients and space on the wood substrate. Thus, modifying the wood environment to favor growth of the biological agent may enhance competitive behavior of the bioprotectant.

4.3.1. Conclusions

B. subtilis # 733 A exhibited promising properties in prevention of wood discoloration, but in situ production of antimicrobial compounds was not noted under the conditions employed. However, assaying for particular antifungal compounds which are known to be produced by particular isolates of B. subtilis [Kugler et al., 1990] or refining the extraction procedure [e.g. solvent] may produce more precise results.

5.0. Assess Spore Germination of Aspergillus niger
BOB on Ponderosa Pine Sapwood in the Presence
of protectant Bacillus subtilis # 733 A

5.1. Introduction

In natural environments, microbial colonization of wood not in ground contact mainly occurs through air - and rainborne inoculum [spores], or is vectored by vertebrates and invertebrates. Successful spore germination and subsequently colonization can be affected by fluctuating environmental conditions [humidity, temperature] and in situ conditions such as wood moisture content, availability of nutrients or the presence of previously established microorganisms.

Most studies of spore germination by wood inhabiting fungi have used microscope slide cultures on agar. Although these techniques allow easy observations, the conditions employed are not representative of the wood surface. A few spore germination studies, however, have used wood-based systems which more closely reflect the natural environment. Morton and French [1966] studied factors influencing spore germination of 3 wood rotting fungi on Douglas-fir and found that incubation temperature, wood moisture and the presence of heartwood decisively affected spore germination. The importance of wood substrate on spore germination of decay

fungi has also been noted by Toole [1971], as has the effect of mass of the wood sample on which the spore lands [Schmidt and French, 1979]. The latter effect most likely reflects the buffering role played by larger wood samples. In situ observations on spore germination of Antrodia carbonica (Overh.) Ryvard & R.L. Gilbertson on Douglas-fir under controlled wood moisture content and temperature conditions indicate that the fiber saturation point represents a threshold for spore germination, while incubation temperature has more variable effects [Przybylowicz and Corden, 1986].

In general, spore germination represents a very critical stage in the life cycle in which the microorganisms are most vulnerable. When spores become hydrated, the plasma membrane is temporarily disorganized and leaks organic substances into the surrounding environment. In the presence of microbial competitors, for instance a biological agent, these substances might be utilized by a resident microflora and subsequent spore germination might be impaired or prevented. Identifying bioprotectants capable of inhibiting germination of spores of stain fungi would represent an excellent strategy for stain prevention.

In most biocontrol studies, inhibition of spore germination of a target species in the presence of a biological agent has been quantified on agar media [Baker et al., 1983; Pusey and Wilson, 1984; Ferreira et al., 1991].

Nutrient media, however, promotes production of antibiotics and thus may not reflect naturally occurring conditions. In this section, a wood-based observation system was designed to quantify in situ spore germination of a target wood staining fungus in the presence of a bioprotectant.

5.2. Material and Methods

5.2.1. Preparation of Inoculum of Bacillus subtilis # 733 A and Aspergillus niger # BOB

A small plug was transferred from a culture tube of B. subtilis 733 A into a 250 ml Erlenmeyer flask containing 50 ml of nutrient broth [Difco]. The flask was then placed on a rotary shaker [80 RPM] and incubated for approximately 60 hours at room temperature [23°C] prior to use.

A. niger was cultured on Potato-dextrose agar [Difco] until abundant sporulation developed. Spores were then flooded off the mycelial mat using a 0.001 % solution of Tween 80 and filtered twice through four layers of cheesecloth. Harvested spores were then drained into a squeeze bottle.

5.2.2. Treatment of Ponderosa Pine Specimens

Steam sterilized [121°C, 10 min] Ponderosa pine [Pinus ponderosa L.] samples [0.45 x 1.4 x 1.5 cm] were dipped into 250 ml of bioprotectant suspension [50 ml nutrient broth culture fluid and 200 ml sterile [distilled water] or into 250 ml of sterile distilled water [controls] for approximately 8 minutes. Samples were then allowed to surface dry [1 to 2 minutes] prior to being sprayed to runoff with A. niger spores.

5.2.3. Incubation Chamber

A small size aluminum incubation chamber [8.2 x 3.0 x 1 cm] with a molded depression [6.6 x 1.4 x 0.5 cm] was employed to observe A. niger spore germination in the presence or absence of the biological agent [Fig. 5.1.]. Wood specimens were placed into this depression and covered with a glass slide. Mineral oil was used to tightly seal off the incubation environment, allowing oxygen to diffuse but denying moisture losses from the chamber as well as preventing the entry of competing microbes. Samples were incubated in room temperature.

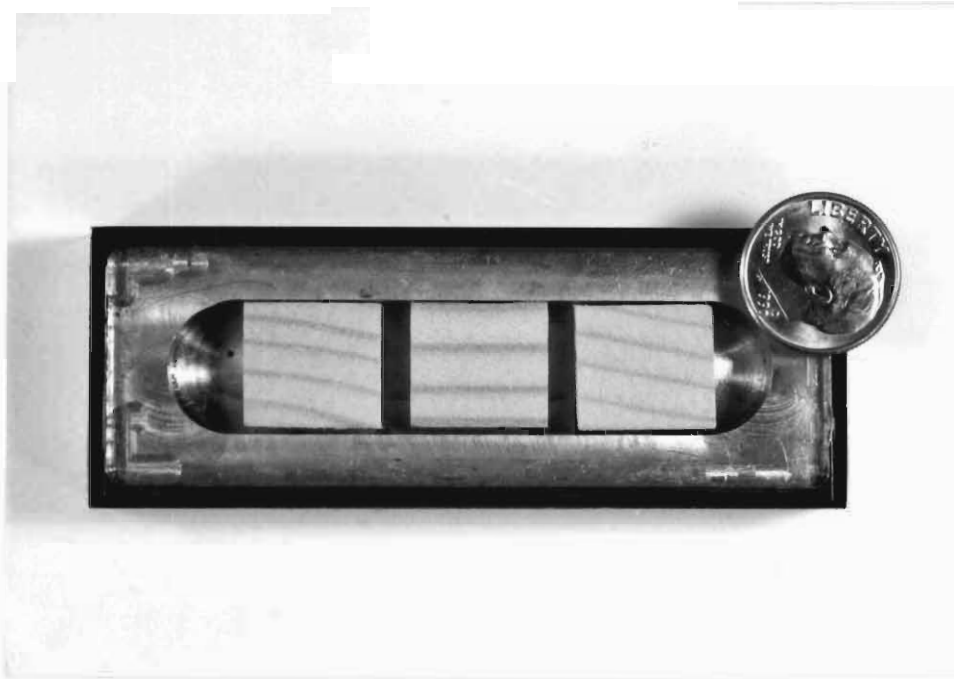


Fig. 5.1.: Incubation chamber used for examination of microbial interactions between B. subtilis # 7 33 A and A. niger on the surface of Ponderosa pine sapwood.

5.2.4. Evaluation of Spore Germination

Incubated wood specimens were removed from the incubation chamber 6, 8, 10, 12, 24, and 36 hours after inoculation and a 20 μm thick section was cut using a Spencer microtome [Spencer Lens Co., Buffalo, N.Y.]. Longer incubation periods were not deemed feasible due to the presence of extensive mycelial growth which limited observations on individual spores. The sections were stained in diluted Safranin followed by steaming in diluted Picro-aniline blue according the procedure described by Wilcox [1964] and mounted in water. Each section was then examined, using a Leitz microscope at 10 x magnification to determine the degree of spore germination as evidenced by production of a 2-3 μm long germ tube. The percentage of spore germination per square centimeter of wood surface area was calculated using the following equation:

$$\% = [(ACG/ACS)/WSA] \times 100 \% \quad [\%/cm^2]$$

where : ASG = mean of three replicates of conidia germination counts at one selected time point.

 ACS = mean of three replicates of conidia sprayed on the wood surface, based on colony forming units produced on PDA.

 WSA = wood surface area of Ponderosa pine specimens.

5.3. Results and Discussion

In situ observations on germination of spores of Aspergillus niger in the presence of bioprotectant Bacillus subtilis # 733 A showed that spore germination was not inhibited under the conditions employed.

Generally, onset of spore germination was divided into uptake of water and outgrowth of a germ tube and was observed as early as 6 hours after inoculation on treated [bioprotectant/A. niger] and control samples. At that time, uptake of water, as evidenced by the presence of some swollen, blue stained spores of A. niger in tracheids and ray parenchyma cell of Ponderosa pine sections, had already occurred. Furthermore, individual spores on both treated and untreated samples had already produced germ tubes, which rarely extended more than 2-3 μm from the spore [Tab. 5.1.]

After 8 to 10 hours of incubation, numerous A. niger spores showed an uptake of water and an outgrowth of germ tubes on both treated and untreated sections. In some instances, however, treated sections displayed swollen spores surrounded by bacteria or had bacteria adhering to them.

Between 10 and 12 hours after inoculation, numbers of successful spore germination increased as did the length of germ tubes; however, observations of close spatial associations between the biological agent and freshly

Table 5.1.:Effect of B. subtilis # 733 A on germination of A. niger conidiospores on Ponderosa pine sapwood at selected time points.

Time [hrs] ^b	% spore germination ^a	
	Control	<u>B. subtilis</u>
6	0.015	0.018
8	0.161	0.167
10	0.272	0.262
12	0.339	0.317

a = as a percent of A. niger spores initially applied.

b = time after inoculation

emerged hyphae were not recorded.

Sections harvested from treated and untreated specimens 12 hours after inoculation showed an increasing number of successfully germinated spores of A. niger, and extensive mycelial growth was observed after 24 and 36 hours of incubation. At the latter time point, branched hyphae were seen to infest several neighboring tracheids. Quantification of spore germination was not possible 24 or 36 hours after inoculation due to extensive fungal growth.

Previous studies have shown that although Eutypa lata ascospores imbibed water, they did not germinate in the presence of an isolate of B. subtilis 72 hours after inoculation [Ferreira et al., 1991]. Production of antibiotics was suggested as a probable mode of action. However, the use of artificial media for this spore germination bioassay differed from the methodology herein employed. Nutritional limitations on wood may not support production of antimicrobial compounds on the wood surface. Although low levels of antibiotic production may have occurred, bacterial cell and fungal spores were observed in close proximity.

Competition for nutrients might have also occurred in cases where a close spatial relationship was observed. Spore germination of Botrytis cinerea Pers.:Fr. on the surface of leaves has been reported to be inhibited by the presence of Pseudomonas spp. isolates and the sequestering of nutrients

from the substrate has been suggested as a probable cause [Campbell, 1989]. Our bioprotectant may have also absorbed organic material from spores of A. niger during breakage of spore dormancy; however, no evidence of detrimental effects on subsequent mycelial growth were noted. Previous studies have demonstrated that spores which leak excessive organic material into the environment during spore germination do not germinate in the presence of a biological agent [Campbell, 1989]. However, close spatial relationships seemed to be necessary for these interactions. Observations made in this study indicate that the biological agent colonized the substrate in clusters, for instance in tracheids, and thus apparently did not uniformly colonize the substrate. Seifert et al. [1987] has also suggested that incomplete colonization by B. subtilis # C 186 produced incomplete bioprotection of Pinus spp. specimens against wood staining organisms.

Although our approach to understand the mode of action of bioprotection of B. subtilis # 733 A on wood staining fungi by using A. niger was not successful, this organism may not be a representative of all mold and stain fungi. In fact, this organism was only chosen because its conidia are easily produced and the size and shape of A. niger spores permitted examination by light microscopy. However, our observations underscore the need to apply both chemical or biological anti-stain treatments within 24 hours after

sawing, because of the very fast growth rates of A. niger. This further highlights that need for biological agents that are highly competitive for both nutrients and space. It may also indicate that strategies which modify the wood substrate in favor of the bioprotectant or sublethal applications of antimicrobial compounds will be essential to increase the ability of the bioprotectant to uniformly colonize the wood substrate and improve the prospects for bioprotection against wood staining fungi.

5.3.1. Conclusions

B. subtilis # 733 A did not inhibit spore germination of the mold fungus A. niger under the conditions employed; however, the wood-based observation system provided a very good method for in situ examination of microbial interaction. The use of a second stain, which preferentially reacts with the bacterium would further enhance observations of spatial relationships between the two organisms. Unfortunately, the destructive sampling procedure prevents prolonged examinations of microbial interactions on the same specimens. The design of a wood-based observation system which permits extended examination over several days of microbial interactions at the wood surface seems to be essential for achieving a better understanding of long-term bioprotection.

6.0.

Conclusions

All the microorganisms tested exhibited bioprotectant potential in previous tests, although not necessarily on wood. While one bioprotectant, B. subtilis # 733 A provided superior protection against wood staining microorganisms, the failure of many of the isolates evaluated indicates that bioprotection may be highly specific for wood species and environmental conditions. It may further suggest that since the majority of the biological agents herein tested are common soil inhabitants, they may be unable to effectively colonize wood, for instance due to nutritional differences between the soil and the wood environment. More microorganisms, particularly initial wood colonizers or benign fungi [Micales and Highley, 1988] need to be screened for their ability to protect unseasoned sapwood from biological stain.

The physiologic condition of the bioprotectant had a significant effect on performance. The results suggest that there is considerable room for improving bioprotectant strategies, for instance through nutritional selections [Benko and Highley, 1991 a+b]. Furthermore, modifying the environment [wood] in favor of the bioprotectant may suppress competing microorganisms and provide improved bioprotection [Dawson-Andoh and Morrell, 1990; Morrell,

1990]. This strategy provides a fertile area for future research.

Despite the obvious nutritional effects which would suggest antibiotic production, no evidence of these compounds was found. Antibiotics may still have been produced in very small quantities or were volatile, but they had little impact on our system [Dennis and Webster, 1971 b]. Alternatively competition for nutrients and space appeared to be a more important mechanism in our system. Spore germination in the presence of the biological agent was, however, not inhibited, suggesting that the bioprotectant was incapable of complete colonization of the wood substrate [Seifert et al., 1987]. Spatial relationships between bioprotectants and other more important wood staining organisms needs more detailed investigation over a longer time period than herein used.

Generally, considerable basic research on areas such as the nutritional aspects of potential bioprotectants as well as the effect of environmental factors on bioprotection are necessary to produce successful bioprotection against staining organisms. Future research should therefore concentrate on the following aspects:

- * Nutritional effects on bioprotection during the life cycle of the bacterium
- * In situ observations on spatial relationships between the biological agent and wood staining fungi

* Effects of environmental factors on growth and effectiveness of the bioprotectant

Nutritional effects on bioprotection also deserve more detailed research. C:N ratio, micronutrient levels, pH and incubation temperature were not addressed in this study but may have important implications on performance. Production of antifungal compounds and competitive properties of B. subtilis # 733 A as a function of incubation time should also receive further study. For example, the activity of antifungal compounds produced in the culture broth of the biological agent may change dramatically at different points during the growth cycle. Identifying production peaks of antifungal compounds as well as competitive properties for nutrients and space as a function of the life cycle of the biological agent would permit application of bioprotectants when prospects for growth are greatest.

The decreases in antagonistic activity of the bioprotectant over incubation time in this study highlight the lack of knowledge about colonization and survival patterns within the wood substrate. These changes may reflect nutritional deficits, deactivation of antifungal compounds by a competitive microflora or environmental conditions unfavorable to the bioprotectant. A more detailed anatomical study of bioprotectant/stain fungus interactions grown under a variety of environmental conditions might

identify spatial relationships which could provide important clues for improving bioprotectant performance.

These results indicate that bioprotection against stain fungi is a viable prospect; however, a far better understanding of the nature of microbial interactions on the wood surface will be necessary before large scale application of this strategy is feasible.

7.0.

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