

Time-temperature schedules to kill wood inhabiting fungi: Proposed test method

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Background

In many countries exporters of lumber or logs are obligated by law to apply approved treatments for phytosanitary purposes. Heating to a core temperature of 56°C for at least 30-minutes (56/30) in a kiln or heating chamber is the most common treatment used for lumber. It has also been accepted as one of the available methods to treat wood packaging material under ISPM15 international standard. This schedule was originally developed to disinfect softwoods that potentially could carry the pine wood nematode *Bursaphelenchus xylophilus* and/or its associated *Monochamus* vector. When the ISPM 15 standard was developed it was recognized that even though this schedule might not kill all organisms, especially heat-tolerant fungi, it would eradicate most pests of concern. Recently Canada and New Zealand researchers have tested temperature/time heat treatment effects on fungi of interest summaries of this work have been presented at the IFQRG meeting in Victoria in February 2005 but the different protocols used have complicated direct comparisons of the results (http://www.forestry-quarantine.org/Feb-2005-Report_e.pdf). It has been recognized that work is needed to standardize test methods and to continue testing other organisms of interest.

Forintek Canada Corp is planning to determine if existing criteria for heat treatment kill the fungi found in trees affected by the mountain pine bark beetle (MPB – *Dendroctonus ponderosae*). In the last decade, the province of British Columbia has witnessed a beetle outbreak that currently affects over 100,000 km² of lodgepole pine forests or over 173,000,000 m³ of standing timber. Given the sensitivity of markets to international transportation of pests and the fact that post MPB-wood is easily identified by the blue color of its stained sapwood, data is required to determine if 56°C/ 30 minutes is sufficient to kill fungal associates of the MPB. As well as various time/temperature combinations, we plan to investigate if air drying and wood aging reduce the viability of embedded fungi or render them heat tolerant (as suggested by the data from New Zealand). This work also aims to refine test protocols and work towards a method that will be used by others to test other fungi. This protocol has been shared with members of the IFQRG for comment and hopefully movement towards a test method we can all use.

Test method

Objectives:

- Determine the effect of selected temperature-time schedules, including 56/30 on the vitality of specific fungi.
- Determine if infested wood and fungi grown on agar slants in vials give the same results
- Determine if wood air-drying (wood aging) alone reduces the viability of embedded fungi
- Determine if slow pre-drying results in heat tolerance in fungi.

Test material

Select an appropriate wood (host) species most suitable for the organism of interest (the pest of interest may not be as well adapted for the standard wood species and might place a fungus at a disadvantage in the wrong wood). For testing mountain pine beetle associated fungi lodgepole pine sapwood, both lab-infested and naturally infested material will be used. Sample dimensions for the time-temperature test are 30 x 10 x 5 mm.

Test organisms (fungi) and replication

Obtain fungal isolates from culture collections or experts (scientists with expertise in the subject ideally at least 3 different strains of each species representing different locations. Once sub-cultured on suitable medium the original isolates must be archived under appropriate storage conditions. Check sub-cultured fungi for stability and vigor, if possible with the experts to determine if the morphology is correct and without colony abnormalities e.g. amoeboid growth and degenerated areas. Pre-test growth on proposed substrates at various moisture contents to determine conditions for optimum growth. For the fungi known to produce chlamydospores (resting spores) make sure to confirm that these spores are present before running the test to assure that the most resistant stage is tested. (See appendix for the list of chlamydospore producing fungi). Replicate each isolate at least 6 times for each treatment. For control treatment use 3 replicates per isolate and submerge them under the water heated at 25°C following required time schedule.

Preparation and inoculation of sterile test material

Use freshly felled, green sapwood of appropriate wood substrate. Prepare 30 x 10 x 5 mm pieces (with grain parallel to the long dimension), and refrigerate in sealed plastic bags. Freeze if they cannot be used promptly. *Note: Bluestain organisms prefer higher moisture contents, but other organisms might have lower optimum moisture contents.*

Prepare cultures for each isolate on 2% MEA and grow until fungi are well established and cover the plate. Note: For slow growing cultures smearing the inoculum with a spreader in a drop of sterile water will produce more growth points and faster plate coverage. These cultures will be used to inoculate the substrate. For isolates grown by central inoculation measure diameter of each after two and four days on two perpendicularly scraped lines on the back of the plates and calculate mean radial increment. Photograph fungal colonies at 7 days using natural light, for comparison with other isolates of the species and to detect changes to recovered fungi.

Prior to inoculation, sterilise test wood. We suggest using following method in the order of preference:

- Gamma irradiation at 2.5 Mrads

- Dipping samples in 70% ethanol for 2-3 minutes and then venting them on a sterile laminar bench for 5-10 min. Quickly pass each sample through a flame to burn off any remaining ethanol. Note: Ethanol sterilisation sometimes may produce a lingering toxic effect.
- Autoclaving for 30 minutes. Note: Autoclaving may change the substrate to the extent that it may not support growth of some fungi.

There may be a need to increase the wood moisture content to support growth of fungi that prefer wet substrates. Sterilized wood can be re-wetted by putting it into sterile water and under vacuum for 3 minutes. Use a sterile desiccator half-filled with sterile water. Submerge samples with sterile inert weights, replace lid and hook to a running water pump to create vacuum.

Place sterilized or re-wetted samples in pre-sterilized breathable-patch bags (they have an air-permeable patch and are designed for mushroom spawn production). Alternatively, use autoclavable bags. These need to be opened once a week under the sterile hood to replenish oxygen. Use 50 g of medium-size, autoclaved vermiculite per 250 test pieces as moisture holding medium and mix it with test wood (Sexton et al, 1993). Wet vermiculite prior to sterilization. Prepare liquid inoculum by pouring a few millilitres of sterile water into the plate containing the culture and slowly scrape aerial portions of the fungus into the water. Transfer the water and scraped mycelium from one plate of the same species into 200mL of sterile water and blend in a sterile blender jar with three short pulses or for 15 seconds only of continuous blend. *Note: For fungi that sporulate prolifically in culture the blending is not necessary. Place liquid into a sterile hand sprayer or use sterile airbrush to inoculate (an option is to use liquid culture to grow inoculum).*

Inoculate by spraying the inoculum of each isolate with a spray bottle separately over the required amount of test samples while agitating them around inside the bag so that all sides are coated. Do not overfill bags, but keep the number of samples per bag such that inoculation is even (for 8 X 16 inch bag we used 250 samples). Keep the samples out of the visible puddles of moisture in the bag. For larger wood sample sizes the use of other inoculation techniques such as placing actively growing mycelia next to or on the top of wood placed on an agar plate may be more appropriate.

Preparation of naturally infested test material

For naturally-infested material, used in parallel, obtain fresh substrate from the field and confirm the viability of embedded fungi by plating small wood splinters onto nutrient and selective media plates. While viability tests are being performed, the naturally-infested material should be stored frozen and cut into sample sizes close to the day of the experiment.

Incubation, monitoring and inspections

Incubate bags with inoculated wood at room temperature (close to 22°C) in a mite free location. Turn the samples as required to stop fungal growth from sticking the samples together. Measure the weight of the bags with samples after the inoculation to monitor if there is significant drop in weight during incubation. The drop may prompt additional water to be added to the system by light spraying over the samples to provide adequate moisture for fungi to grow. There should always be a sign of condensation on the inside of the bags.. Incubate for 3-6 weeks until colonisation is complete. Open bags carefully to allow oxygen to be replenished at one-week intervals (if you are not using breathable patch bags). *Note: Less time is required for stain and some mold fungi, longer for the decay fungi. Samples inoculated with bluestain were incubated for 3-4 weeks prior to heat treatment while basidiomycetous fungi for 6-8 weeks. Sacrifice a few wood pieces to check if fungus*

thoroughly colonized it. Where appropriate, check for formation of chlamydo spores to assure that the most resistant stage of the fungus is tested. For parallel tests with the same fungi grown on agar slants in vials, grow fungi two weeks prior to the experiment.

Heat treatment

Heat test samples that are fully colonized with test fungi, or the naturally infested samples, in a water bath to a matrix of seven different temperatures for four different treatment times. The method is a slight modification of the test method developed for testing wood infested with pinewood nematodes (Task force on pasteurization of softwood lumber, 1991, see also Newbill and Morrell, 1991). The following complete time /temperature matrix will be used:

Time (minutes)	Temperature °C						
	41	46	51	56	61	66	71
<1							
30							
60							
120							

Note: Control consists of a set of three samples for each isolate. Put samples through the same handling process, but “heat treat” by placing bags in water at 25°C followed by fungal viability as described below. The time/temperature range is simply bracketing the 56/30 criteria by 5C intervals. For other work temperature ranges may be extended or decreased, depending on the literature or expert opinions for particular fungi. 30, 60, 75, 90 min (as used in previous Canadian and New Zealand work)

Place inoculated wood into sterile vacuum-sealable bags, (all 6 replicates for one temperature treatment of one isolate in one bag). Aseptically arrange in a single flat layer, without samples touching, in a polyethylene film bag. Pull the film tight to the specimens and hold specimens in position while vacuum sealing the bag. This will facilitate heat transfer to the wood.

Note: If this is not done the remaining air will act as an insulator and the required temperature might not be achieved.

Mark identification of samples on outside of bag. Sandwich bag in between two mesh grids (metal or plastic) for submersion and easy recovery.

Note: Before submersion conduct preliminary tests to determine the required time to reach target temperature and cool-down times at the core of the wrapped samples at different temperatures. Do this by inserting thermocouples into a one mm diameter hole drilled 15 mm to the centre of the several sacrifice blocks from the end and stapled in place. Similarly insert thermocouples into the vials with agar through a hole made on the vial’s lid. Seal the surface of the hole with silicone caulking or expanding urethane foam to prevent entry of the air. Calibrate required time of immersion accordingly to meet target heat time. The pre heating and cooling time will differ for different wood species, temperatures and wood moisture contents, as well as if you use glass or plastic vials so it needs to be calibrated for each case. From the PWN work it was found that required heating time is ~4 minutes. From earlier CFS/Forintek work that studied 8 pathogenic fungi there was some variation at different temperatures between different species of wood (e.g. pine took 1.8 to 3 minutes while oak 3-3.8 min to heat to target temperature. In our preliminary tests it took 1.6 min for completely dried lodgepole pine wood to reach the target

temperature. For freshly inoculated wood it took 2.7 minutes at temperatures 66 and below and 3.6 minutes at 71C. Thus in our experiment we allowed 4 minutes for pre-heating before starting to count time. Agar dispensed in Simport (T 406-2 12 ml) polystyrene vials took longer to heat (8.5 minutes) compared to agar in Fisher (333726) 7 ml borosilicate glass (4.5 min). We routinely allowed 4.5 minutes for pre-heating of agar in the vials before starting to count time.

Submerge bag containing test wood in a hot-water bath 2 cm off the bottom at desired temperature and for desired time. Tighten and wrap the lid of each vial individually with parafilm to prevent water from getting into the vial. Submerge vials under the water but allow the lids to remain above the water surface. Monitor and record the temperature of the bath during the immersion. *Note: In our preliminary studies the temperature dropped for 0.2 C immediately following sample submersion but come back quickly to target temperature within 40 seconds.*

Remove wood and vials from hot water and cool by immersion in 25°C water. *Note: It took a few seconds for temperature to drop 5 C below target temperature and up to 2 minutes to drop 15°C below target temperature.* Immediately following cooling, loosen vial caps to allow oxygen in as well as break the vacuum seal by slicing the bags to allow air back into the bags with the wood test specimens. Subculture both wood and vials to assess the survival.

Air drying of wood and fungal survival

Prepare, inoculate and incubate the required number of samples so that there is set of three replicate wood samples inoculated with each isolate. Remove each set of three replicates per isolate specified times to attempt fungal recovery. Air-dry samples on grids in sterile containers covered with autoclaved natural cotton batting or cotton flannel and keep in an environmental chamber set to obtain equilibrium moisture content of 15% (say 76% RH at 20C at sea level). Check viability of fungi by destructive sampling (as described below) after 0, 4, 8, 12, 16 days, and 3, 4, 5, 6, 7, and 8 weeks and 4 months.

Air drying (desiccation) and heat-tolerance

To determine whether slow desiccation will change the heat tolerance of the test fungi air-dry sets of inoculated samples on grids in sterile containers as described above for the air drying and keep in an environmental chamber set to obtain equilibrium moisture content of 15%. Store samples under these conditions for 15, 40 and 90 days, then subject to heat treatment as described above and proceed with the viability checking.

Assessment of survival and recovery procedure

The day after heat treatment or after air-drying treatment, place test piece aseptically on 1% MEA Petri-plates to grow out any surviving fungi. *Note: We examined if there was a difference in recovery of fungi on regular 1% MEA compared to moist host sawdust agar and found no differences. Therefore, for simplicity agar is recommended. (To make sawdust agar use 100 g of milled sawdust from debarked host twigs or sapwood in 1000 ml of water. Add 30 g of agar, autoclave for 30 min at 121C and 15 PSI. Dispense in plates while constantly agitating).* Attempt fungal recovery from agar slants by plating two plugs taken from across the colony onto fresh 1% MEA.

Monitor subcultured samples regularly, for fungal outgrowth. Check at 7 and 21 days for any outgrowth and note any unusual features of growth. If feasible subculture the ones that

survived, and compare colony morphology and growth rates to the original culture to determine whether the test time/temperature affected the fungus. The identity of an isolate can be confirmed by using molecular methods or by employing vegetative compatibility (VC) trials (ability of hyphae to anastomose and form single unit) where a recovered isolate is paired on an agar plate with the original isolate, allowed to grow, and then the interaction zone between the two studied. If isolates are the same, this results in colonies merging or showing very slight mycelial thickening at the junction. Incompatibility results from genetic differences, and often shows different types of vc reactions in the zone of mycelial proliferation where the two colonies meet. The vc reactions have been described in a number of fungi, but some preliminary tests might be needed to study chosen test isolates and the most appropriate agar medium to enhance the reaction.

References:

- Newbill, M.A. and Morrell, J.J. 1991. Effect of elevated temperatures on survival of Basidiomycetes that colonise untreated Douglas-fir poles. *Forest Prod. J.* 41(6):31-33.
- Sexton, P.G., Forsyth, P.G. and Morrell, J.J. 1993. A comparison of agar exposure and vermiculite burial methods for preparing basidiomycete-colonized wood. *Material und Org* 28:39-46
- Smith, R.S. (ed.). 1991. The use of heat treatment in the eradication of the pinewood nematode and its vectors in softwood lumber. Report to the Task Force on pasteurization of Softwood Lumber. Forintek Canada Corporation, Vancouver, B.C., Canada. 72 pp.
- Stalpers, J.A. 1978. Identification of wood-inhabiting aphylophorales in pure culture. *Studies in Mycology* No. 16. 248 pp

Appendix 1. Basidiomycete fungi that produce chlamydospores (from Stalpers, 1978)

1. *Abortiporus biennis* (Bull. ex Fr.) Sing.
2. *Amylocystis lapponica* (Romell) Bond. & Sing.
3. *Anomoporia bombycina* (Fr.) Pouzar
4. *Antrodia carbonica* Overh.
5. *Antrodia malicola* (Berk. & Curt.) Murr.
6. *Antrodia oleracea* Davidson & Lombard
7. *Antrodia serialis* (Fr.) Murr.
8. *Antrodia sinuosa* (Fr.) Sarkar
9. *Antrodia vaillantii* (DC. ex Fr.) Cooke
10. *Antrodia xantha* (Fr. ex Fr.) Cooke
11. *Bjerkandera adusta* (Willd. ex Fr.) P. Karst.
12. *Bjerkandera fumosa* (Pers. ex Fr.) P. Karst.
13. *Bondarzewia berkeleyi* (Fr.) Bond. & Sing.
14. *Bondarzewia montana* (Quel.) Sing.
15. *Ceraceomyces borealis* (Romell) J. Erikss. & Ryv.
16. *Ceriporia alachuana* Murr.
17. *Ceriporiopsis rivulosa* (Berk. & Curt.) Cooke
18. *Climacocystis borealis* (Fr.) Imaz.
19. *Climacodon septentrionalis* (Fr.) P. Karst.
20. *Veluticeps fimbriata* (Pers. ex Fr.) Pouzar
21. *Daedalea quercina* (L.) ex Fr.
22. *Dichomitus squalens* (P.Karst.) D. Reid
23. *Dichostereum effuscatum* (Cooke & Ellis) D.P. Rogers & H.S. Jacks.
24. *Dichostereum pallescens* (Schw.) D.P. Rogers & H.S. Jacks.
25. *Diplomitoporus lindbladii* (Berk. & Br. ex Berk.) Cooke
26. *Echinodontium tinctorium* Ellis & Everh
27. *Fistulina hepatica* (Schaeff.) ex Fr.
28. *Fomitopsis cajanderi* (P. Karst.) Kotl. & Pouzar
29. *Fomitopsis meliae* Underw.
30. *Fomitopsis officinalis* (Vill. ex Fr.) Donk

31. *Fomitopsis palustris* Berk. & Curt.
32. *Fomitopsis pinicola* (Schw. ex Fr.) P. Karst
33. *Fomitopsis spraguei* Berk. & Curt.
34. *Ganoderma colossus* (Fr.) C.F. Baker
35. *Ganoderma lucidum* Boud. apud Pat.
36. *Gloeocystidiellum porosum* (Berk. & Curt.) Donk
37. *Gloeophyllum abietinum* (Bull. ex Fr.) P. Karst.
38. *Gloeophyllum odoratum* (Wulf. ex Fr.) Imaz
39. *Gloeophyllum protractum* (Fr.) Imaz.
40. *Gloeophyllum sepiarium* (Wulf. ex Fr.) P. Karst.
41. *Gloeophyllum striatum* (Sw. ex Fr.) Murr.
42. *Gloeophyllum trabeum* (Pers. ex Fr.) Murr.
43. *Grifola frondosa* (Dicks. ex Fr.) S. F. Gray
44. *Hapalopilus croceus* (Pers. ex Fr.) Donk
45. *Hapalopilus mutans* Peck
46. *Hericium coralloides* (Scop. ex Fr.) S.F. Gray
47. *Hericium erinaceus* (Bull. ex Fr.) Pers.
48. *Hymenochaete rubiginosa* (Dicks. ex Fr.) Lev.
49. *Hyphodermella corrugata* (Fr.) Bres.
50. *Hypochnicium vellereum* (Ell. & Cragin) Parm.
51. *Hypochnicium vellereum* (Ell. & Cragin) Parm.
52. *Inonotus rickii* (Pat.) D. Reid
53. *Laetiporus sulphureus* (Bull. ex Fr.) Bond. & Sing.
54. *Laxitextum bicolor* (Fr.) Lentz
55. *Megalocystidium lactescens* (Berk.) Boidin
56. *Melanoporia nigra* (Berk.) Cooke
57. *Microporellus obovatus* Berk. & Curt.
58. *Mycoacia fuscoatra* (Fr.) Donk
59. *Osteina obducta* (Berk.) Donk
60. *Perenniporia compacta* Overh.
61. *Perenniporia fraxinophila* (Peck) Ryv.
62. *Perenniporia robinophila* (Murr.) Lloyd
63. *Phaeolus schweinitzii* (Fr.) Pat.
64. *Phanerochaete chrysosporium* Burds. & Eslyn
65. *Phanerochaete sordida* (P. Karst.) Burt
66. *Phlebia merismoides* Fr.
67. *Phlebia subserialis* H. S. Jacks. & Dearden
68. *Phlebia subserialis* (Bourd. & Galz.) Donk
69. *Phlebia tremellosus* Schrad. ex Fr.
70. *Phlebia chrysocreas* (Berk. & Curt. apud Berk.) Burdsall
71. *Piptoporus betulinus* (Bull. ex Fr.) P. Karst
72. *Polyporus brumalis* (Pers. ex Fr.) Fr.
73. *Polyporus mori* (Bosc.) ex Fr.
74. *Poria aurea* Peck
75. *Postia amara* (Hedgec.) Lowe
76. *Postia balsamea* (Peck) Murrill
77. *Postia placenta* (Fr.) Cooke
78. *Postia salmonicolor* (Berk. & Curt.) Pouzar
79. *Postia sericeomollis* (Rom.) Bond. & Sing.
80. *Postia tephroleuca* (Fr.) Donk
81. *Punctularia atropurpurascens* (Berk. & Br.) Petch
82. *Pycnopus cinnabarinus* (Jacq. ex Fr.) P. Karst
83. *Pycnopus sanguineus* (L. ex Fr.) Murr.
84. *Radulodon casearium* Ryv.
85. *Schizophyllum commune* Fr.
86. *Sparassis crispa* (Wulf. ex Fr.) Fr.
87. *Spongipellis delectans* (Peck) Murr.
88. *Spongipellis pachyodon* (Pers.) Kotl. & Pouzar
89. *Spongipellis unicolor* (Schw.) Murr.
90. *Sporotrichum pruinosum* Novobranova
91. *Trametes cubensis* (Mont.) Sacc.
92. *Trametes pubescens* (Schum. ex Fr.) Pilat
93. *Trametes suaveolens* (Fr.) Fr.
94. *Trametes versicolor* (L. ex Fr.) Pilat
95. *Tryomyces chioneus* (Fr. ex Fr.) P. Karst
96. *Tryomyces fissilis* (Berk. & Curt.) Donk
97. *Tryomyces fumidiceps* Atk.

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98. *Vararia granulosa* (Fr.) Laurila
99. *Veluticeps berkeleyi* (Berk. & Curt.) Cooke