Video Article

Agar-Block Microcosms for Controlled Plant Tissue Decomposition by Aerobic Fungi

Jonathan S. Schilling[1], K. Brook Jacobson*[1]

[1]Department of Bioproducts and Biosystems Engineering, University of Minnesota

*These authors contributed equally

Correspondence to: Jonathan S. Schilling at schilling@umn.edu

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Abstract

The two principal methods for studying fungal biodegradation of lignocellulosic plant tissues were developed for wood preservative testing (soil-block; agar-block). It is well-accepted that soil-block microcosms yield higher decay rates, fewer moisture issues, lower variability among studies, and higher thresholds of preservative toxicity. Soil-block testing is thus the more utilized technique and has been standardized by American Society for Testing and Materials (ASTM) (method D 1413-07). The soil-block design has drawbacks, however, using locally-variable soil sources and in limiting the control of nutrients external (exogenous) to the decaying tissues. These drawbacks have emerged as a problem in applying this method to other, increasingly popular research aims. These modern aims include degrading lignocellulosics for bioenergy research, testing bioremediation of co-metabolized toxics, evaluating oxidative mechanisms, and tracking translocated elements along hyphal networks. Soil-blocks do not lend enough control in these applications. A refined agar-block approach is necessary.

Here, we use the brown rot wood-degrading fungus *Serpula lacrymans* to degrade wood in agar-block microcosms, using deep Petri dishes with low-calcium agar. We test the role of exogenous gypsum on decay in a time-series, to demonstrate the utility and expected variability. Blocks from a single board rip (longitudinal cut) are conditioned, weighed, autoclaved, and introduced aseptically atop plastic mesh. Fungal inoculations are at each block face, with exogenous gypsum added at interfaces. Harvests are aseptic until the final destructive harvest. These microcosms are designed to avoid block contact with agar or Petri dish walls. Condensation is minimized during plate pours and during incubation. Finally, inoculum/gypsum/wood spacing is minimized but without allowing contact. These less technical aspects of agar-block design are also the most common causes of failure and the key source of variability among studies. Video publication is therefore useful in this case, and we demonstrate low-variability, high-quality results.

Video Link

The video component of this article can be found at https://www.jove.com/video/2283/

Protocol

This protocol applies to woody and non-woody substrates, as outlined, as well as to oven- or air-dried material. Read through the protocol first, however, before set-up. There are several points raised that may apply to your study, and these points (underlined) require planning. Also, note that there are two published agar-block methods that are occasionally used, one the British Standard 838 and another following an International Research Group on Wood Protection (IRG-WP) paper submitted by Bravery (1978). Our resembles method 838, with modifications primarily in the microcosm design and the control of the agar medium, but again, both approaches are often avoided due to historic moisture control issues in wood blocks, causing anoxia and variability. A good review of these test methods that includes discussion of agar-block designs, including the 838 standard, can be found in Nicholas (1973).

1) Preparing Microcosms

Microcosms for these trials are 1 cm taller (deeper) than typical petri dishes, increasing head space above wood blocks. They are filled with a modest and exact amount of agar in order to control absolute nutrient amounts, in addition to their concentration, and to keep wood blocks well away (>3 mm) from the lid. The agar used in this case of gypsum testing is a low-calcium Type A agar; however, we show representative results using Blakeslee's medium, the ATCC recommended medium for maintaining the test isolate of *Serpula lacrymans* (Wulfen: Fries) Schroeter strain EMPA 65 (ATCC 32750).

This design keeps plant tissues away from agar contact and away from the dish lid and walls. Variable wetting of lignocellulosic substrates is the key source of variability in agar-block tests. Wetting to increase moisture content creates anoxia and suppresses or even halts aerobic biodegradation. It also creates a problem for anyone studying oxidative mechanisms of brown and white rot fungi responsible for wood decomposition. Condensation on plate lids is an issue if free water droplets form and wet the substrate. Likewise, wood and other tissues will

'wick' water rapidly from agar when in contact, leading to moisture contents in excess of 80% (dry wt. basis) and halting aerobic degradation. Tissues must be distanced from these water sources, allowing the filamentous fungus to find, connect, and control moisture within the substrate.

1. Make enough agar media to fill the desired number of petri plates with 20 ml agar, each. We use five replicates (n=5) per treatment, determined by power analysis using past results.
2. For calcium- and iron-free Type A agar, we add 15 g agar to a 500 ml volumetric flask containing approximately 400 ml deionized water amended with 1.0 g NH_4NO_3, 1.0 g monobasic KH_2PO_4, 0.25 g $MgSO_4$ x $7H_2O$, and 1.0 g glucose. To the mixture, we use stock solutions to add micronutrients. We add 50 µl each of H_3BO_4 (0.057 g / 100 ml) and $ZnSO_4$ (0.031 g / 100 ml). We add 50 µl each of $MnCl_2$ (0.036 g / 100 ml), $CuSO_4$ (0.039 g / 100 ml), and $(NH_4)_6Mo_7O_{24}$ (0.018 g / 100 ml). Once nutrients are added, fill the volumetric flask to the 500 ml line.
3. A typical calcium addition in this case would be 0.05 g $CaCl_2$ x $2H_2O$ / 500 ml. In our case, we use agar calcium as a treatment, with a 5 mM final concentration. We counter the increase in ionic strength and chloride addition by adding 5 mM NaCl to the other microcosms. Similarly, we use iron-free media for this demonstration, but in cases where iron is included, we mix 0.112 g of $FeSO_4$ with 2 ml deionized water and add 50 µl of this fresh solution (not stock) immediately after vortexing. In any media where you control additions of nutrients, it is wise to test pH before autoclaving. Acidic or basic additions (eg. $FeCl_3$) will affect solidification.
4. Transfer media to a flask and autoclave the medium at 121°C and 16 psi for 20 minutes. We do not exceed 500 ml volumes (per 1000 ml flask), to avoid having agar solidify before we can administer all of it to the test plates.
   (Note: It is wise when using alternative media, especially minimal nutrient agar with added basal salts, to first verify that your test fungus will grow on it. High ionic strengths can inhibit growth or even kill your test isolate.)
5. Use a portable pipette-aid and 10 ml sterile polystyrene pipettes to transfer agar aseptically in a biosafety cabinet, once flasks cool. Letting media cool to touch is important here to minimize condensation. Also, stack plates high as they are poured, to minimize free water on the lids. While condensation is a nuisance in normal culturing, here it represents a major problem if droplets form and wet the wood. Moisture contents (MC) over 80% (dry weight basis) will create anoxia in tissues, limit decay by aerobic fungi, and increase variability. Calculate MC as follows:
   MC* = [(Fresh weight x dry weight) / dry weight] x 100
   *(MC can exceed 100% - this may seem an odd way to calculate MC, but is standard)
   A. Alternative microcosms: If plant tissues are tested which must be pre-milled and sieved (eg, corn stover stalks and leaves, together), there are two dish options we use. Divided petri plates are available with 2 or 4 sections, and agar can be excluded from compartments with powder. Solidified agar can also be cut and a portion removed to leave room for powder, but care must be taken to maintain equal agar volumes remaining if nutrient availability is to be controlled.

6. Add generously cut plastic mesh grids to the plate surface, using thoroughly washed and autoclaved grids added aseptically. We use a product, Gutter Guard (35 x 50 mm, 2 mm thick), for our mesh, and have used scanning electron microscopy to show a clean surface after soap and water wash and to show a lack of fungal penetration. We have had mixed success with glass fiber filters and with laying blocks directly on developed mycelia, both due to wicking issues. You will get decay, but your coefficient of variability (CV) will be high, making treatment comparisons statistically weak. We used glass rods previously, but blocks are susceptible to sliding off rods when jostled, leaving blocks in contact with agar. One good alternative is to cut complete circles to fit the plates, cutting one edge off to accommodate inoculum. In general, cut grids to fit your own set-up, but make sure they lay completely flat in the petri dishes.

2) Preparing 'Block' Substrates

These protocols have been developed for solid wood, but are adaptable for other plant tissues. Mass loss is the standard measure for decay progress in wood degraded by filamentous fungi. Thus, our approach uses oven-dry weights pre- and post-decay to determine mass loss. However, for any bioenergy research, where the focus is on the plant tissue chemistry, many find that air drying tissues is preferable. We show here protocols for preparing your agar-block cultures to use oven-dried starting material, but give the alternative information to air dry and also to process powder instead of solid substrates.

1. For this demonstration, we use Southern Yellow Pine (SYP). SYP is a commercially-available lumber representing the vast majority of lumber used in residential housing in the U.S. It can be any one of four *Pinus* species. Non-treated lumber is used and knot-free blocks are cut from a single rip (longitudinal cut) along the grain (19 x 19 mm). This minimizes chemical variability in the wood. This length is cut into 19 mm^3 blocks. We use this size for soil-block trials, and cut many blocks in a single session on the table saw.
2. The 19 mm^3 blocks to be used in agar-block microcosms are further split in half along the grain, using a chisel and hammer, not a saw. This makes one rough block edge to face down on the plastic mesh, and a smooth top edge to label. Label blocks with pencil. If you cannot label your substrates, be sure to devise a system to keep up with samples. Cut enough blocks to satisfy your treatments (again, we use n=5 per treatment), as well as non-inoculated controls that will serve both as contamination monitors and as baseline data samples, conditioned in parallel.
3. For oven-dried material, place samples in a convection oven at 100°C for 48 hr. If air-dried material is required, condition the samples in a chamber or room with constant humidity and temperature. We use 65% RH and 20°C, and we usually count on 10-14 days conditioning, depending on the material.
4. Pre-weigh your samples to determine initial oven-dry or fresh weight. With samples from the oven, simply transfer samples to a desiccator to cool and weigh them.
   A. Alternative air-drying: With air-dried material, take five samples (n=5; sacrificial these will not be used in microcosms), weigh them fresh, oven-dry them as above, and re-weigh after drying. Calculate a moisture correction factor for each of two blocks as follows:
   MC correction factor = dry weight / fresh weight
   Example: 2.46 g (dry) / 2.68 g (fresh) = 0.918; so, a 3.0 g fresh block is 2.75 g oven-dried
   Average the five samples to obtain the average correction factor. Then, weigh all of your air-dried blocks. Multiply each weight by the average MC correction factor to determine initial oven-dry weights.

5. Autoclave labeled samples at 121°C and 16 psi for 1 hr, or longer with larger samples. Tightly foil to minimize wetting.

A. Alternative sterilization: Gamma radiation can be used to sterilize, if available. We have tested spruce and beech wood previously for effects of temperature on hemicellulose loss, and found none, along with no strength loss or color change. However, this may differ among substrates and your preferences/needs, and gamma radiation is a proven alternative.

6. For this demonstration, we are testing the role of solid gypsum (pure versus 1% $FeSO_4$) on degradation of our pine (SYP) samples. These are made as discs with exact surface areas and volumes, again to control their availability in addition to their concentration. These are autoclaved separately and added during the inoculation step. We need one disc for every block, and we are adding two blocks per petri dish to allow two harvests.

3) Inoculating & Labeling

Inoculating agar-block microcosms is more time-consuming than inoculating soil-block jars. For us, we count on each inoculation taking 3 min. To petri dishes containing agar, this is the point of addition for mesh, wood, any exogenous nutrient sources (here, gypsum pellets), and the fungus. There is increased chance for contamination because of the amount of time the lid is open and the number of visits inside. There are also several key mistakes that are commonly made at this stage, and these are best covered by coupling video with text. Watch the video.

1. In a sterile biosafety cabinet, assemble your empty agar dishes, a source plate for your fungal inoculum (we use 2 wk cultures), mesh, plant substrate (wood), exogenous materials, a sharpie, parafilm strips, and transfer tools. We flame sterilize using 70% ethanol, and we use a transfer tool for inoculum and forceps for blocks and mesh. Parafilm should be cut with a razor to avoid any nicks on the edges. Nicks in parafilm lead to breaks when sealing, and this will jostle samples and require re-entry.
2. Flame transfer tools or forceps before adding the following (in this order):
   A. Plastic mesh.
   B. Wood substrate. For our demonstration, we will add two wood blocks along the length of the mesh, both from the same initial block that was split so we can pair data from the early and late harvests. These blocks are added side by side, with the grain end (wood cross section) facing the point of the source of fungal inoculum.
   C. Exogenous materials. Here, we are testing the role of gypsum on decay by a filamentous fungus. Therefore, we want the fungus to encounter these gypsum discs before reaching the wood, and thus add one gypsum disc between each inoculum point and block on top of the mesh. This means adding 2 discs per microcosm. Do not allow contact between discs and the wood, but keep them close.
   D. Fungal inoculum. We use a #4 cork borer with 7 mm diameter to make plugs from 2-wk cultures grown in 20 ml agar. This helps to control inoculum volume. For non-inoculated controls, it is best to add a plug from a sterile plate. We typically add these plugs to the agar, and not on the mesh. Do not allow contact between inoculum and either the exogenous substrates or the wood, but again, place them close together. There is one inoculum plug per block.
   Note: It is wise to assemble these contents in an agar dish before starting to ensure there will be at least 3 mm of head space, that there will be at least 3 mm distance between wood blocks and the lid walls, and that your plastic mesh dimensions will easily accommodate your substrates.
3. Parafilm dishes to seal them, keeping an alcohol flame burning and with forceps ready. Hold plates horizontal and wrap parafilm in a smooth continuous motion. If parafilm breaks or if contents jostle, remove parafilm, re-enter and assemble contents, and try again.
4. Label the dish lids using an alcohol-resistant marker, labeling block numbers directly over top of respective substrates. We also draw circles over the gypsum discs. As wood decays, you will likely lose the ability to read labeling on the substrates. It is important to keep up with dish position. Also, do not underestimate the ability for a fungus to degrade or colonize myriad of other material types, including metals.
5. If you have delicate assemblage on your mesh, transfer plates CAREFULLY to the incubator. We have, only once, bumped the bin of plates against a door jam and had to re-assemble the plates. Take your time, and plot your route. It is a one-time concern, so take care this one time.

4) Incubating & Harvesting

Plates can be incubated to suit your fungus, but should be kept in a biological incubator if possible, to avoid condensation due to temperature fluctuations. Time series harvests are done aseptically, except for the last harvest. If these intermediate harvests are done after growth on the substrates is significant, handling the plates is easier because hyphae cross link substrates.

1. For our demonstration, we incubate plates at 20°C and in the dark. At a week 5 harvest point, we remove the entire treatment lot, spray each bottom and top with 70% ethanol and use flamed forceps to remove material inside the biosafety cabinet.
2. Take pictures before destroying agar plates, focusing on any morphology or melanization.
3. Remove blocks to be treated as your needs require. Use your finger to roll away excess hyphae (with nitrile gloves), but be careful not to lose any decayed material. We usually oven-dry blocks in aluminum weigh pans to determine mass loss, using fresh and dry weights of controls to monitor for excessive wicking. We also label and track any dark brown blocks that are clearly water-logged, although this should be minimal following this approach. Mass loss data helps gauge decay progress, and it is important data if elemental concentrations are to be calculated on a gram-weight basis, later. Also remember that percentage data must be normalized for statistics. We calculate as follows:
   % Mass loss = [(initial weight final weight) / initial weight] x 100
   A. Alternative air-drying: If you require air-drying, with your goal to biologically treat the material further, you should use the conditioning regime from Section 2.3 to recondition. If you must determine mass loss, it will require a measure of moisture content, One solution is to split blocks (or powders) into a large and small portion. Weigh both, but only oven-dry the small portion. Calculate moisture content conversion factor, as above, and then apply it to the total fresh weight of small + large portions (total block fresh weight). Especially if pretreating material with a fungus prior to saccharification or other processes, mass loss will help you with mass balance determination later and accounts for carbohydrates consumed by the fungus.

4. Destroy the cultures in autoclavable bags, but save plastic support mesh and any other components that can be recycled for use in future experiments.

5) Interpreting Results

Lignocellulosic tissues will usually decay slower in agar-block designs, but at this point you should have relatively low variability even at moderate decay levels. You should also have moderate (20-50%), not high moisture in control tissues.

1. Moisture contents (on a dry weight basis) in wood not exposed to fungus are typically moderate, around 40% in this demonstration. This is above the fiber saturation point (FSP) of pine (normally around 24%), which means there is some free water in excess of the water bound within the lignocellulosic secondary cell wall. This also means that some wicking likely occurs. An RH of 100% should still not allow wood MC to exceed the FSP. Your design may achieve different results depending on substrate and mesh selection. The key is to keep moisture contents below 85% or so, on a dry weight basis.
2. The % mass loss in wood degraded in these agar-block microcosms is typically around 30% after 16 weeks for most fungi. For select fungi, in this case Serpula lacrymans, fungi will achieve full degradation in this time,>60% mass loss. S. lacrymans is a brown rot fungus, removing little lignin, so 62% mass loss in this trial means decay is near or past completion. White rot fungi will remove lignin, and mass loss depends on species as well as substrate.
3. Take care to record hyphal morphology. In this demonstration, hyphal morphology did not inherently show success or failure in any one treatment, but the melanization colors were important. It allowed us to relate the role of iron as an impurity in gypsum to older studies like Low *et al.* (2000), where native materials were tested and where this 'rust' colorization was observed. The role of calcium and iron were debated in Low *et al.* (2000), and here we see enhanced decay with iron, not calcium, and these same rust-colored hyphae.
4. If you want to measure concentrations of anything in this degraded wood, it is best to normalize for mass loss. If, for example, calcium is neither imported or exported from wood decayed 50%, its concentration on a weight basis will appear to double from time zero to the final harvest. That is because 1 g of powder now represents twice the wood volume than it did at time zero. The fungus consumed 50% of the matrix, decreasing density. Normalize any given concentration (for example mmol/g) to compensate for mass loss as follows:
   Normalized concentration = mmol/g x [1 - (% Mass loss / 100)]
   Example: 10 mmol/g Ca in wood degraded 20%, versus 7 mmol/g at time zero.
   Question: Did Ca content increase during decay?
   10 mmol/g is an apparent 3 mmol/g increase, a 43% increase, but one gram of powder from degraded wood with lower density now represents a larger volume. Normalization is required to compare initial and final Ca content in equal wood volumes.
   10 x [1 x (20% / 100)] = 8 mmol/g normalized
   Answer: Yes, Ca did increase, but by 14%, not 43%.
   (Data can also be expressed per wood volume, mmol/cm^3, by multiplying by density.

Figure 1. Agar-block microcosm, as set up in this demonstration, before incubation.

Figure 2. *Serpula lacrymans* colonizing pine wood blocks resting on plastic mesh to elevate blocks above agar-contact. This mycelium represents an important connection between wood and exogenous nutrient/element sources, and controlling these exogenous material sources in the agar or as solid materials is a key advantage of agar-block design, versus soil-block design.

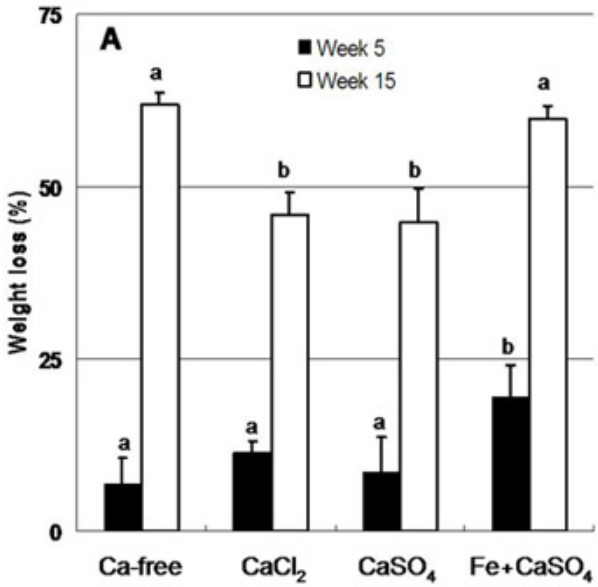

Figure 3. Mean % weight loss, as a measure of extent of wood decay by *Serpula lacrymans* after 5 and 15 weeks incubation with pine in agar-block microcosms. Treatment were none (Ca-free), 5 mM $CaCl_2$ added to agar ($CaCl_2$), >99% pure gypsum ($CaSO_4$), or 1% iron-amended gypsum. Protected ANOVA means comparisons were using Tukey's tests, with α=0.05. For each harvest, bars under the same letter are not significantly different. Error bars = standard deviation. Published in Schilling (2010).

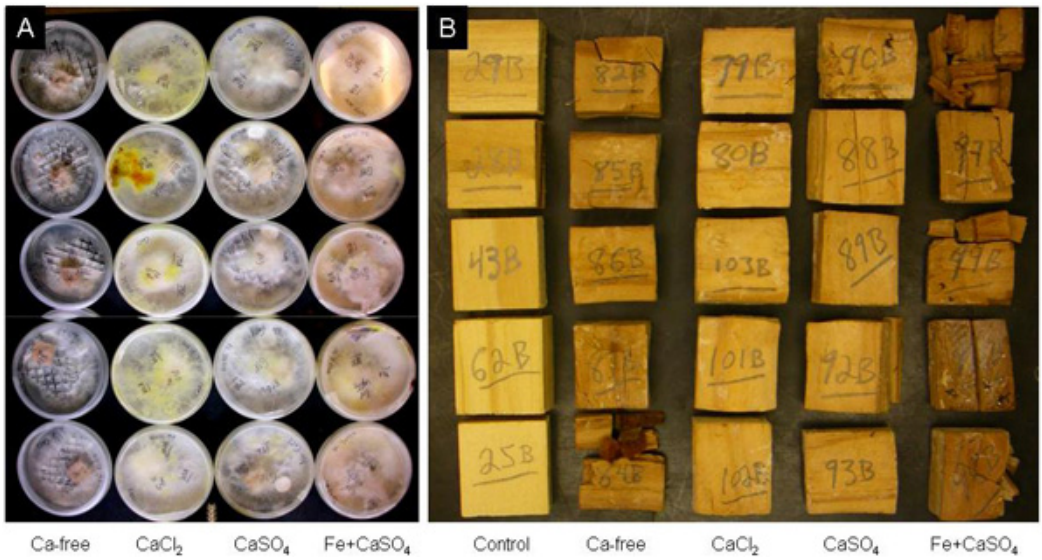

Figure 4. Overhead picture (A) of all five replicates at week 15 of decay by the same brown rot test fungus, Serpula lacrymans, as well as the blocks (B) removed and oven-dried. Note the lack of melanization in Ca-free treatment, the yellowing in pure calcium treatments, and the rust appearance in iron-amended treatment. Treatments are labeled as in Figure 3, with the control in (B) being non-inoculated blocks for comparison.

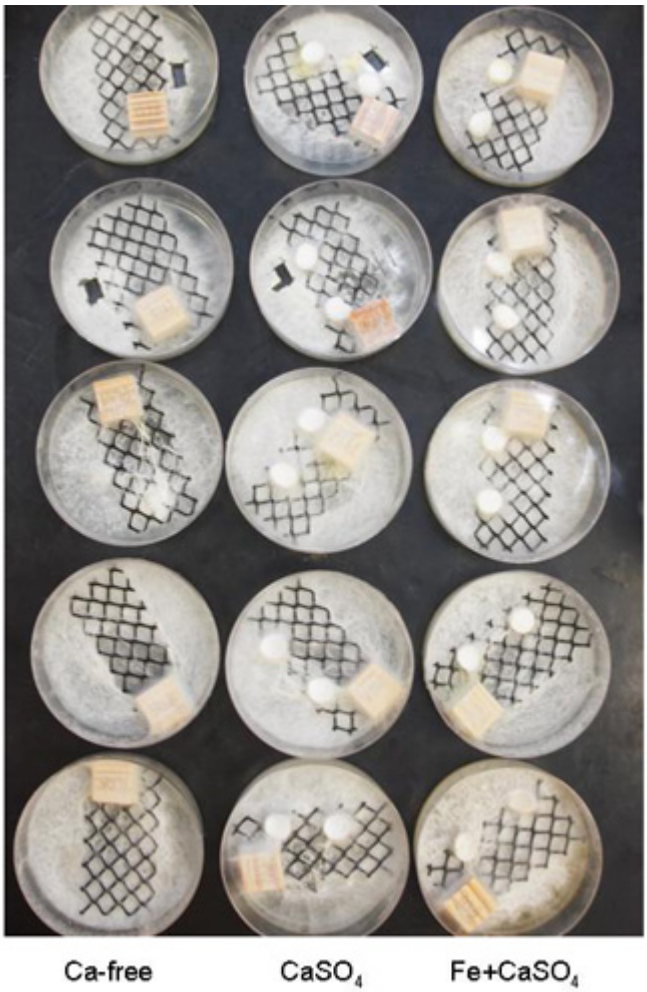

Figure 5. Overhead picture from a different trial using a higher iron concentration medium, Blakeslee's malt agar. Note the loss of observed melanization, compared with Figure 4. Blocks removed and weighed showed no treatment effect on weight loss. This is presented as a

demonstration of the influence of exogenous components of these block trials. These effects would not be testable in soil-block jar designs, where these exogenous inputs are too difficult to control.

Discussion

Using our agar-block set-up (Figure 1) *Serpula lacrymans* grew in direct contact with the gypsum surfaces and into wood blocks (Figure 2), leading to more than 60% weight loss in the control brown-rotted pine blocks (Figure 3). This easily satisfies the ASTM standard goal of >50% decay, and the average coefficient of variation (C_V) in decay at was 0.055 at week 16. This data is published in Schilling[7]. Again, other fungi will require longer incubation in agar-block than in soil-block. For reference, we have successfully run similar designs in several past experiments with a variety of other fungal species, often using scanning electron microscopy with electron dispersive spectroscopy (SEM-EDS) and inductively coupled plasma spectroscopy (ICP-OES) to verify connectivity with exogenous substrates and translocation of calcium and other elements into wood[8,9].

In addition to low variability, there are two other results that our agar-block design reveals. First, there was a treatment effect (Figure 3), where our calcium additions, whether to the agar as $CaCl_2$ or as pure gypsum, inhibited decay by this fungus. This is a useful result because others have theorized calcium enhances decay based on studies with real-world materials like mortar and plaster. Instead, we see decay rates rebound with the addition of iron to the gypsum, suggesting iron, not calcium, is key. The second useful result, however, is not quantified but is instead the color of the mycelia (Figure 4). There are significant and obvious melanization differences in external hyphae among treatments, and researchers have previously noted 'rust' melanization when encountering this fungus on building materials (eg. Low*et al.* 2000). In our more recent studies, we found that these effects are lost using a high-iron medium (Figure 5). Collectively, it suggests that this fungus utilizes iron, not calcium, in these materials and that earlier studies, with 'rusty' mycelia reported, were observing iron effect, not calcium effect.

Overall, these results and a lack of treatment effects using high-iron agar are a very strong demonstration of the control that the agar-block design can provide the researcher, especially in light of the alternative soil-block approach.

Disclosures

No conflicts of interest declared.

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