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Cellulose nanomaterials in growth media for wood decay fungi

Katie Ohno, Rachel Arango, Ronald Sabo, Craig Clemons, Grant Kirker, Amy Bishell
USDA Forest Service
Forest Products Laboratory
One Gifford Pinchot Drive,
Madison, Wisconsin, 53726, USA

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Katie M Ohno[1], Rachel A Arango[1], Ronald Sabo[1], Craig M Clemons[1], Grant T Kirker[1], Amy B Bishell[1]

[1] USDA Forest Service, Forest Products Laboratory, One Gifford Pinchot Drive, Madison, Wisconsin, 53726, USA
katie.m.ohno@usda.gov, rachel.arango@usda.gov, ronald.sabo@usda.gov, craig.m.clemons@usda.gov, grant.kirker@usda.gov, amy.b.bishell@usda.gov

ABSTRACT

Cellulose nanofibrils (CNFs) were investigated as a partial substitution for agar in growth media for wood decay fungi. Radial growth measurements of eight basidiomycete fungi were taken on solid growth media with and without CNFs. Additionally, fungal strain virulence was evaluated using the European CSN Standard EN 113-2 wood decay durability test. The inclusion of CNFs did not significantly affect fungal growth rates on media or in wood decay tests. Rheological evaluations showed that gels from standard malt extract agar were significantly stronger and stiffer than those prepared by a two-thirds substitution of agar with CNFs. However, CNFs were found to add additional stiffness with one-third of the standard amount of agar compared to agar only growth. The solid growth media produced with CNFs appear to be adequately strong and stiff for fungal radial growth and manipulation for wood decay experiments but has some challenges in terms of mixing and processing.

Keywords: Basidiomycetes, agar, cellulose nanofibrils, rheology

1. INTRODUCTION

Laboratory cultures of basidiomycete wood decay fungi are used in standardized tests to evaluate durability of various wood materials, products, and preservative treatments. Growth media for culturing wood decay fungi typically consist of agar as a gelling agent in combination with various additives, including sugars, as nutrients to support fungal growth. Malt extract agar (MEA) is one type of media that is commonly used to culture basidiomycete wood decay fungi. MEA is water soluble and provides carbon, protein, and nutrients to support fungal growth; it also contains 1.5% agar to solidify the media. Agar is an ideal gelling agent as it is stable at a wide range of temperatures (dissolves above 85 °C, solidifies between 32-42 °C upon cooling), non-toxic, metabolically inert, and not easily digested by most organisms. However, one growing challenge is that natural sources of algae (*Gelidium* spp., *Gracillaria* spp. and *Pterocladia* spp.) for producing agar are being over exploited, prompting evaluations of other materials that may serve as gelling agents (Basu et al., 2015; Das et al., 2015).

Cellulose nanofibrils (CNFs) can be produced chemically or mechanically from any cellulosic plant material (Moon et al., 2011). CNFs are highly biocompatible (can be used in various medical applications) and have properties that include transparency, high viscosity (i.e., gelling agents), and high strength (i.e., reinforcement for composites) (Siro et al., 2010). CNFs may offer an alternative or partial substitute for agar as a gelling agent in fungal growth media (Schwarze et al., 2022). Adapting CNFs to match the desirable attributes of agar-based growth media is a challenge,

specifically, that it can be sterilized, and pipetted/poured into Petri dishes at elevated temperatures, and solidifies into a relatively strong and uniform gel at room temperature.

Here, we evaluated the application of CNFs as a gelling agent in fungal growth media and the resulting effects on fungal growth and decay rate. The goal of the latter was to determine whether the performance and attributes of the CNF mixtures were suitable for growing laboratory cultures of wood decay fungi. The impact of this research includes the identification of a more sustainable alternative material to traditional microbiological agar. As gelling agents are used across a diversity of scientific disciplines, this work may also have implications for the use of CNF-agar based composite gels in other fields (e.g., biochemical, medical applications) (Drakos et al., 2021). Additionally, this research could lead to improved methods for fungal growth and maintenance which could influence research into protection strategies that mitigate degradation of wood products by these organisms.

2. EXPERIMENTAL METHODS

2.1 Fungal Strains

Five brown rot, one dry rot, and two white rot type basidiomycete fungi were selected for testing including, *Coniophora puteana* (Schumach.) P. Karst. isolate ME-715, *Rhodonia placenta* (Fr.) M.J. Larsen & Lombard isolate MAD-698, *Gloeophylllum trabeum* (Pers.) Murrill isolate MAD-617-R, *Wolfiporia cocos* (F.A. Wolf) Ryvarden & Gilb. isolate MD-106-R, *Fibroporia radiculosa* (Peck) Parmasto isolate FP-90848-T, *Serpula lacrymans* (Wulfen) J. Schrot. isolate ATCC 36335, *Trametes versicolor* (L.) Lloyd isolate MAD 697, and *Irpex lacteus* (Fr.) Fr. isolate HHB-7328, respectively. Initial inoculum was obtained from fungal cultures maintained on agar slants filled with sawdust which were stored at 4 °C to retain vigour. Fungi were subcultured up to 4 times on malt extract agar (MEA) (i.e., 1.5% malt extract broth (BD Biochemical, NJ) + 1.5% bacto agar (BD Biochemical) on Petri dishes (100 x 20 mm) (Fisher Scientific, MA) grown at 27 °C and 70% relative humidity (RH) in the dark. Plugs of fungal inoculum (5 mm dia.) were taken from the growing edge of approximately seven-day old cultures to start experiments.

2.2 Production of Cellulose Nanofibril Materials

Cellulose nanofibril materials (CNFs) for this study were produced in the USDA Forest Service Forest Products Laboratory pilot plant by TEMPO ((2,2,6,6-Tetramethylpiperidin-1-yl)oxyl) oxidation of bleached Kraft pulp under alkaline conditions as described elsewhere (Reiner and Rudie, 2017; Saito et al., 2007). TEMPO catalyzed oxidation results in charged surfaces on the fibers which facilitate charge repulsion and result in a strong gel compared to other CNFs. The CNFs used in this study have a carboxylate surface content of 1.5 mmol/g and a solids content of 0.99%.

2.3 Radial Growth on Cellulose Nanofibril Media

For cellulose nanofibril growth media (CNFA), CNFs were used to replace two-thirds of the agar in solid media as follows: 0.5% agar and 1.5% malt extract broth were added to 1% CNFs suspended in di-H2O and compared to standard media (MEA) consisting of 1.5% agar and 1.5% malt extract broth. These suspensions were mixed well using either a mix-stir agitator rod (CNFA) or a magnetic stir bar (MEA) and then sterilized by autoclave at 121 °C for 20 min. After sterilization, suspensions were again mixed either by stir plate (MEA) or by swirling vigorously (CNFA). Thirty grams of media was pipetted into Petri dishes (100 x 20 mm) (Fisher Scientific, MA), for a final media depth of 5 mm. Two separate experiments were conducted with triplicate Petri dishes containing one plug per fungus per media type (n=6). Growth measurements were made by taking two perpendicular diameters every two days for eight days, with a 12-day measurement added for slow growing strains. The exception was *W. cocos,* which was measured

every day for four days due to rapid radial growth. Average radial growth was calculated for each plate as follows: RA = (D1+D2)/4, where, RA is average radial growth, D1 is the measured diameter 1, D2 is the measured diameter 2 (which is perpendicular to D1).

2.4 Fungal Strain Virulence

After the radial growth assay, plugs of each of the fungal strains (5 mm dia.) were transferred directly onto CNFA or MEA as the base culture media in deep Petri dishes (100 x 25 mm) (Fisher Scientific, MA), in order to evaluate virulence in a modified European CSN Standard EN 113-2 (2020) standard durability test. Both types of media were mixed and sterilized as previously stated in section 2.3 and pipetted to a final depth of 5 mm (~30 g media). Fungi were allowed to grow completely to the edge of each dish before wood blocks were added. Wood cubes (10 mm^3) of southern pine (*Pinus* spp.) and sweetgum (*Liquidambar* spp.) from a random stock were conditioned in 27 °C and 30% RH and then weighed to the nearest mg. Three cubes of each wood species were placed on top of sterile gutter guard cut to a diameter of 100 mm to slightly elevate test specimens from the growth media. Testing was run in duplicate dishes for each strain of fungus on either CNFA or MEA plates (n = 6). Petri dishes were sealed with parafilm and maintained in a growth chamber at 27 °C/70% RH in the dark for 8 weeks. At the end of the test period, blocks were removed from dishes, gently brushed to remove fungal mycelia and oven dried overnight at 60 °C. Weight loss was calculated after blocks were re-conditioned at 27 °C/30% RH for one week.

2.5 Rheological Tests

To investigate the effects of CNFs on the rheology and gelation behaviour of the growth medium, we used small amplitude oscillatory shear (SAOS) protocols similar to those developed by Mao et al. (2016) for investigating agar gelation. Measurements were performed on an MCR 302 Compact Modular Rheometer (Anton Paar USA Inc.; Ashland, Virginia, USA) using a parallel plate geometry with a 25.4 mm diameter. Temperature was controlled by a Peltier unit with a hood.

For rheological measurements, growth media were prepared by first mixing the suspensions with a polytetrafluoroethylene (PTFE) stirring impeller in a double boiler for one hour, followed by stirring on a hot plate at a setpoint of approximately 100 °C for the duration of rheology experiments. For the MEA solutions, 100 g of deionized water was added to a 250 mL Erlenmeyer flask with 1.5 g malt extract broth and either 1.5 g or 0.5 g of agar. For CNFA, 100 g of CNF suspension (at 1.0% solids) were added to a 250 mL Erlenmeyer flask with 1.5 g malt extract broth and 0.5 g of agar.

Hot solutions/suspensions were transferred to the rheometer, with plates preheated to 70 °C. A nominal initial gap of 0.5 mm was used, and evaporation was minimized by applying a thin layer of mineral oil around the sample and using a hood. After allowing 15 minutes for temperature equilibration, the temperature was decreased at a constant rate of 1 °C/min to 20 °C and then was held for approximately 2 hours to allow gelation to complete. All measurements were made at 1 Hz, 0.01% strain, and a normal force of 0 Newtons. All results were replicated. After gelation experiments, amplitude sweeps (0.01-1% at 1Hz) were performed on the resulting gels.

Various rheological parameters were measured throughout the test, including storage (G') and loss (G") moduli, shear stress and strain, as well as changes in the gap between the parallel plates. Plate gap measurements were adjusted for the effects of thermal expansion of the measuring head based on separate tests on water.

2.6 Statistical Analysis

Results from the radial growth and fungal virulence plate assays were evaluated in a Mann-Whitney's U test to determine statistical significance using PAST statistical software (Hammer et al., 2001).

3. RESULTS AND DISCUSSION

3.1 Radial Fungal Growth

All decay fungi evaluated were able to grow on CNFA plates. Average radial growth of the white-rot decay fungi grown on MEA and CNFA plates are shown in Figure 1. Differences in radial growth as determined by Mann-Whitney analysis at $p \leq 0.05$ are given in Supplementary Tables 1 and 2 for white and brown-rot fungi, respectively. Results showed *I. lacteus* grew equally on MEA and CNFA media, while *T. versicolor* showed a small, but significant increase in growth on MEA over CNFA through day 8.

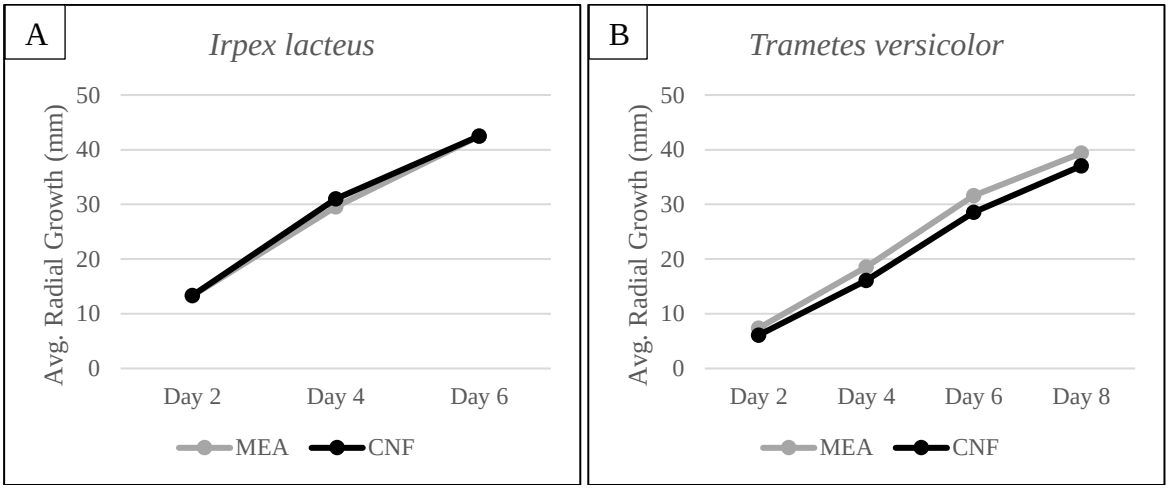

Figure 1: Average radial growth of A) *Irpex lacteus* and B) *Trametes versicolor* grown on MEA plates (gray) and CNFA plates (black).

Average radial growth of the brown-rot (*C. puteana*, *G. trabeum*, *F. radiculosa*, *R. placenta*, and *W. cocos*) and dry rot (*S. lacrymans*) decay fungi grown on MEA and CNFA plates are shown in Figure 2. Results suggest that most fungi grew similarly on both types of media. Three fungi, *G. trabeum*, *R. placenta*, and *W. cocos* showed slightly increased growth on MEA over CNFA. However, *S. lacrymans* showed significantly higher growth on CNFA over MEA by day 8 (Figure 2E, $p = 0.03$).

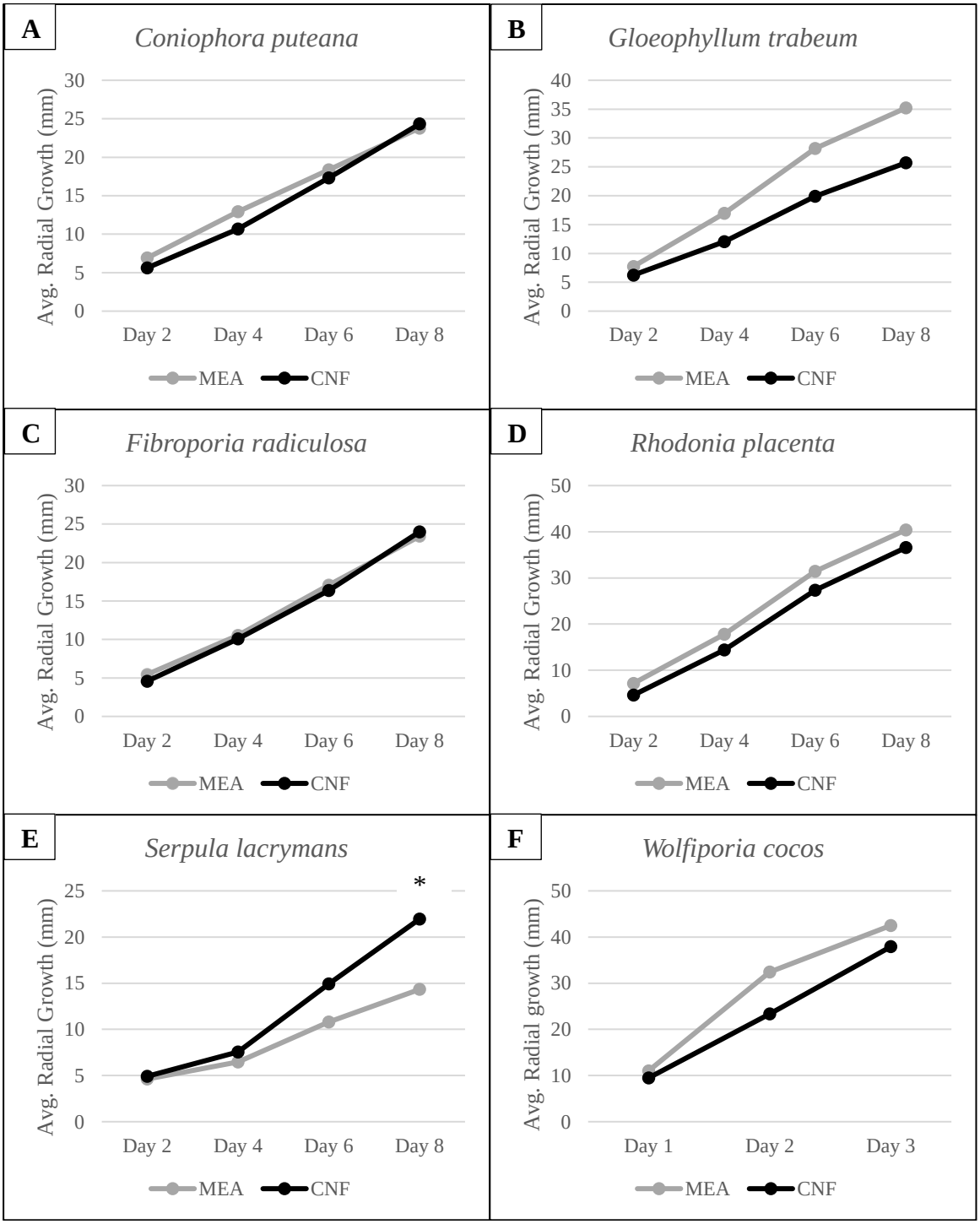

Figure 2: Average radial growth of A) *Coniophora puteana*, B) *Gloeophyllum trabeum*, C) *Fibroporia radiculosa*, D) *Rhodonia placenta*, E) *Serpula lacrymans*, and F) *Wolfiporia cocos* grown on MEA (gray) and CNFA (black) plates. Significant differences within fungi are denoted by astericks.

In general, all fungal strains tested showed comparable growth on CNFA as on MEA suggesting that incorporating CNFs into fungal growth media will not drastically impact growth. While there do appear to be slight differences in radial growth for specific decay fungi grown on MEA vs. CNFA, these differences should be confirmed in further tests with additional replicate samples.

3.2 Fungal Virulence

Results of the modified EN 113-2 decay test of mini wood blocks on CNFA or MEA plates are shown in Figures 3 and 4 for white- and brown-rot fungi, respectively. Differences in decay as determined by Mann-Whitney analysis at $p \leq 0.05$ are given in Supplementary Table 3. This test was aimed at determining if the underlying growth media (i.e., CNFA or MEA) fungal virulence on wood sample blocks. White-rot fungus, *Irpex lacteus*, showed an obvious preference for sweetgum wood over pine and caused a significantly higher amount of decay to sweetgum in association with CNFA compared to MEA (Figure 3). Results showed decay of pine wood by brown-rot fungus, *G. trabeum*, to be significantly higher in association with CNFA media, while pine wood decayed by brown-rot fungus, *W. cocos*, was significantly higher in association with MEA media.

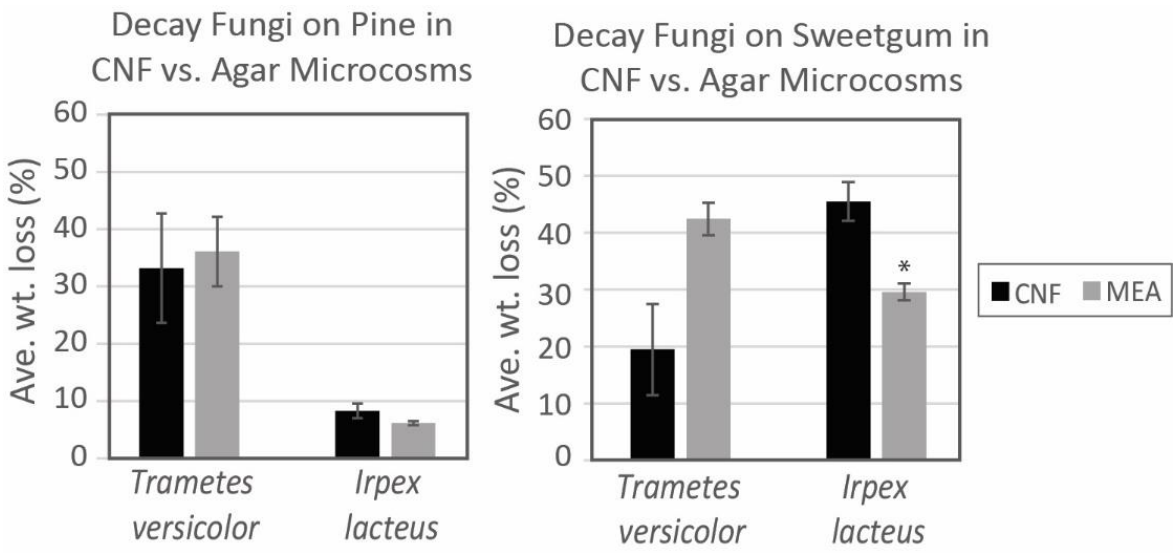

Figure 3: Decay (% weight loss) of southern pine (left) and sweetgum (right) exposed to white-rot fungi, *Trametes versicolor* and *Irpex lacteus* grown on MEA (gray) and CNFA (black). Error bars represent standard error; significant differences within fungi are noted by an astrisk ($p \leq 0.05$) (note: comparisons were only made for decay on CNFA vs. MEA within individual fungi, no comparisons were made for differences between fungi).

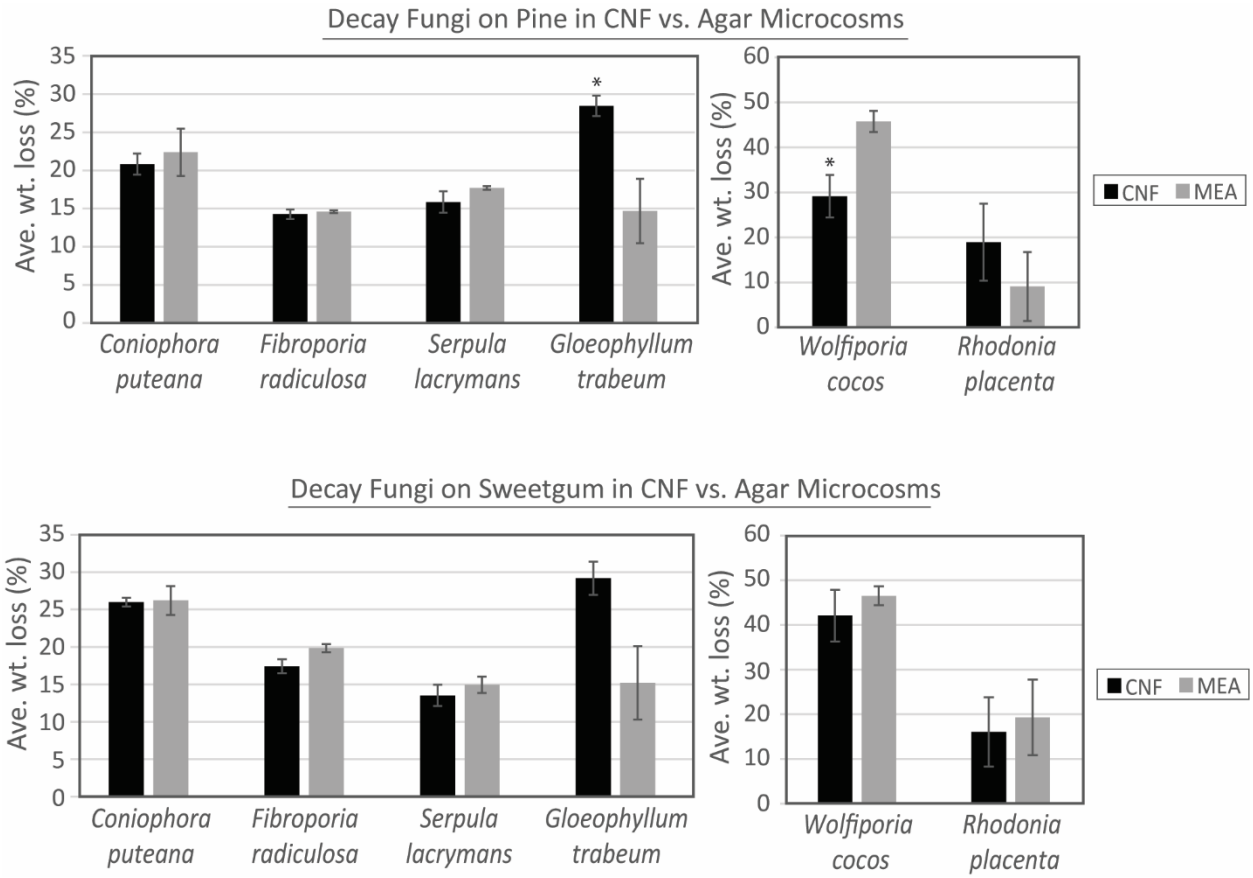

Figure 4: Decay (% mass loss) of southern pine (top) and sweetgum (bottom) exposed to brown-rot and dry rot fungi: *Coneophora puteana*, *Gloeophyllum trabeum*, *Fibroporia radiculosa*, *Rhodonia placenta*, *Serpula lacrymans*, and *Wolfiporia cocos* grown on MEA (gray) and CNFA (black). Error bars represent standard error; significant differences within fungi are noted by an astrisk ($p \leq 0.05$) (note: comparisons were only made for decay on CNFA vs. MEA within individual fungi, no comparisons were made for differences between fungi).

In a study on a similar type of material, Schwarze et al. (2022) evaluated micrfibrillated cellulose (MFC) as a growth media substrate for wood decay fungi. They found that *Daedalea quercina* (heart rot), *Ganoderma adspersum* (white-rot), *Ganoderma lipsience* (white-rot), *Kretzchmaria deusta* (white-rot), *Pycnoporus cinnabarinus* (white-rot), and *Trametes pubescens* (white-rot) were able to grow on approximately 30 g of 3% MFC growth media although, the rate of fungal growth was significantly reduced compared to MEA (Schwarze et al., 2022). However, these authors did show a substainal improvement in fungal growth when yeast or malt extract was added to MFC media (Schwarze et al. 2022). Additionally, they showed that *D. quercina*, *G. lipsiense*, *P. cinnabarinus*, and *T. pubescens* grew more rapidly on 3% MFC + malt compared to malt alone. They concluded that MFC combined with a carbon source can be used for culturing wood decay fungi (Schwarze et al., 2022). MFCs used in that study were produced by mechanical fibrillation of wood pulp and differ from the CNFs used in this study. The CNFs used here have carboxylate charge groups on the surface, and compared to MFC, they have more uniform nanoscale diameters, are more transparent, and are more viscous at a given concentration.

Overall, our results show that all wood decay fungi grew similary on CNFA media when compared to MEA with the exception of *S. lacrymans*, which showed significantly higher growth on CNFA media. This could be a result of *S. lacrymans*' ability to translocate nutrients (i.e., cellulose) which could support growth on alternative growth mediums (Hastrup 2005). In the fungal virulence tests, *G. trabeum* caused significantly higher decay on pine when grown in CNF media. This could be

attributed to *G. trabeum*'s ability to utilize readily availabe cellulose but this should be confirmed through additional tests especially since the other brown-rot fungi did not follow the same trend.

3.2 Rheological Tests

Much can be learned about gelation and the behaviour of the resulting gels through rheological testing. Several different formulations containing various amounts of agar, malt extract, and CNFs were examined by SAOS tests using parallel plate geometry. Prepared solutions/suspensions were slowly cooled from 70 to 20 °C and then held isothermally for 2 hours to complete gelation. Figure 5A summarizes the changes in storage (G') and loss (G") moduli of the standard 1.5% agar plus 1.5% MEB. The rapid increase in G' relative to G" indicates gelation, which started after about 35 minutes, when the temperature reached approximately 35 °C. Ultimately, a G' value of over 30 kPa was achieved after gelation. This behaviour is typical of such agar solutions (Mao et al., 2016).

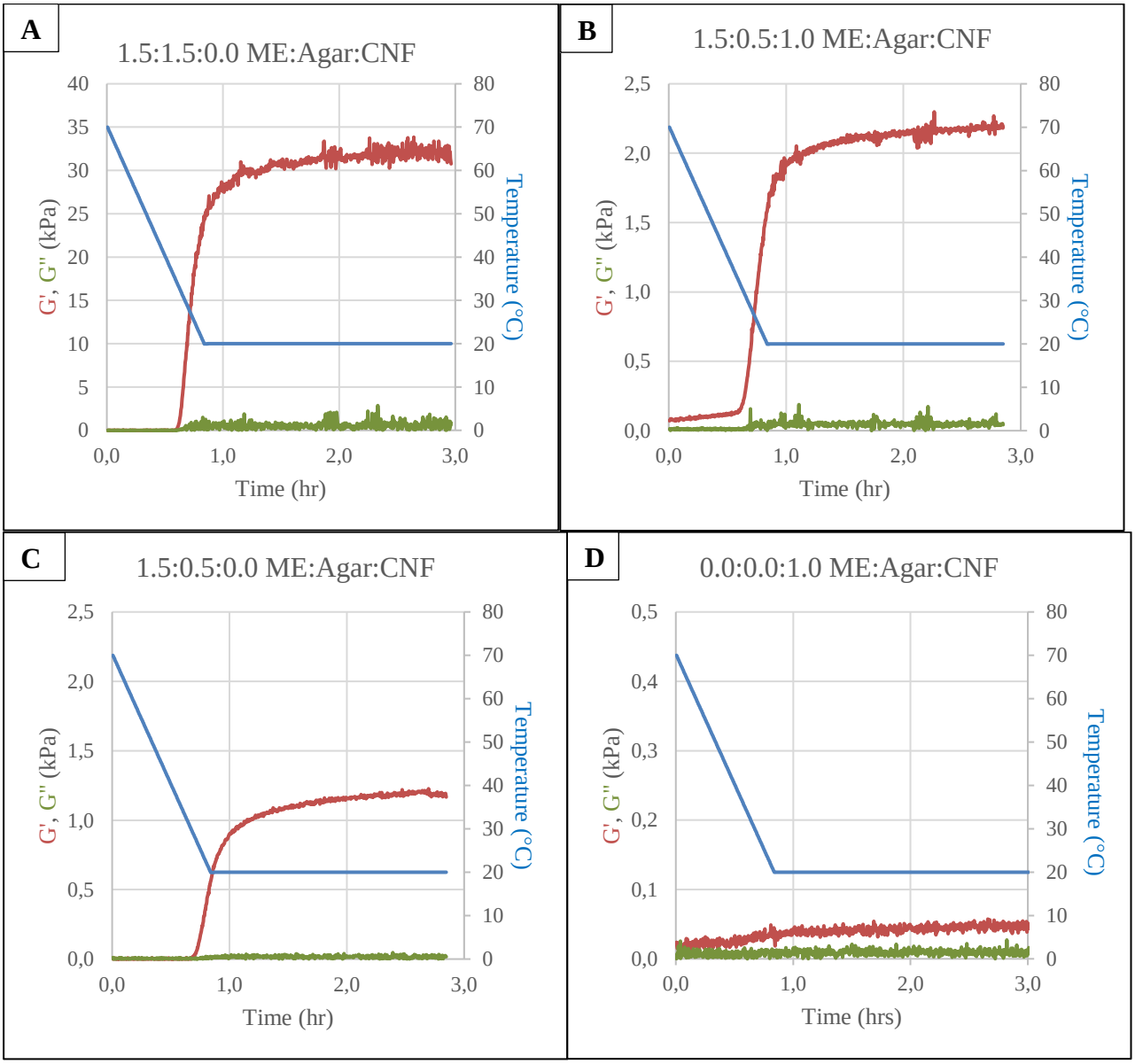

Figure 5: Gelation behaviour of solution/suspension formulations as measured by small amplitude oscillatory shear (SAOS) tests. Numbers in the graph titles are weight ratios.

Replacing two-thirds of the agar with CNFs (i.e., CNFA) drastically changed gel behaviour (Fig. 5B). While the temperature of the onset of agar gelation changed very little, G' after gelation was an order of magnitude smaller, indicating a much softer final gel. Also, unlike the other dispersions, G' was slightly elevated at the beginning of the test, suggesting some slight initial gel structure, well below the temperature at which agar typically gels.

To separate the effects of less agar and CNF addition, we also prepared a solution with the lower agar content (0.5%) but no CNFs (Fig. 5C). Interestingly, although replacing two-thirds of the agar with CNF (i.e., CNFA) led to an order of magnitude decrease in G', it was still almost double that of the similar solution without the CNF at the low agar content. Most of the increase occurred near the agar gelation temperature found for the standard MEA. Figure 5D shows the slow and slight increase in G' over the course of the rheological test for a 1% CNF suspension.

The change in gap size during the rheology tests are shown in Figure 6. No change was found in any of the suspensions after the first hour of testing, presumably because there was no further temperature reduction, and the gel structure was sufficiently developed to prevent shrinking or swelling. For the standard MEA (blue curve), the gap size did not change until the onset of gelation, and then decreased by about 3.5% during gelation. This is consistent with trends found by others (Mao et al., 2016). CNFs alone (top curve) do not undergo thermogelation, and the gap only increased slightly (~1%) during testing.

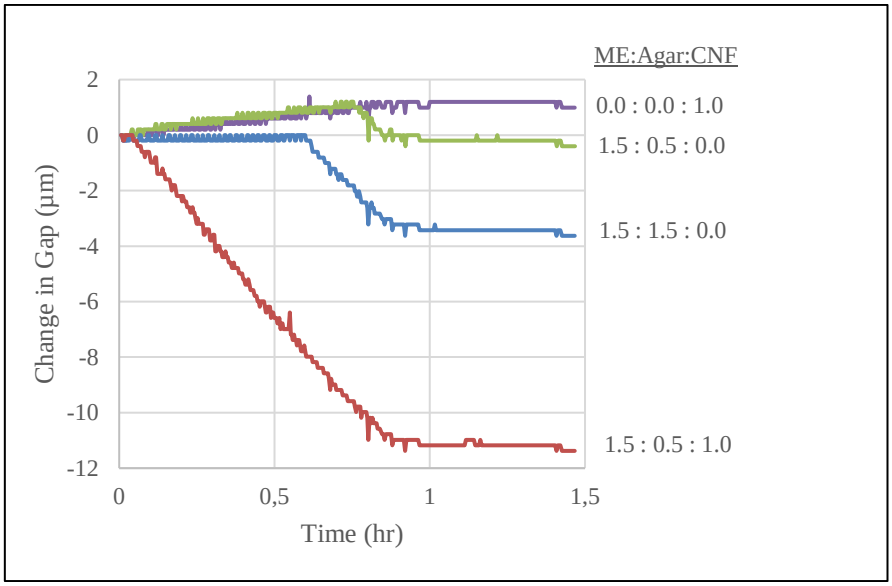

Figure 6: Comparison of shrinkage/expansion of solutions/dispersions during rheological testing. Numbers in the legend are weight ratios.

Interestingly, when two-thirds of the agar gelling agent was removed (green curve), the suspension swelled slightly with decreasing temperature prior to gelation; the standard MEA did not. The onset of gelation was also delayed slightly relative to the standard MEA. The gap reduction due to gelation was approximately proportional to the amount of agar removed.

However, what was most surprising is that with the CNFA gel (bottom curve), a large reduction (~11%) in the gap was found over the course of the test and gelation did little to disturb the downward trend. This behaviour is very different from that of the other solutions/suspensions. While much more research would need to be performed to understand this behaviour, it suggests that the mechanism for structure formation for CNFA gel is quite different than in the other solutions/suspensions.

At the end of the gelation experiments, amplitude sweeps were performed on the resulting gels to better understand how they fail. The storage moduli (G') from the tests are shown in Figure 7. The sudden and precipitous drop in G' for the gel made from the standard MEA solution indicates brittle failure. For CNFA, the resulting gel failed more gradually, which is common for soft gels. The low agar content MEA gel demonstrated an intermediate brittleness but failed at higher strains than the standard MEA gel. As with G', the maximum shear stress achieved just prior to failure for the standard MEA was an order of magnitude higher than those of the low agar content MEA gel, and the CNFA gel (graphs not shown).

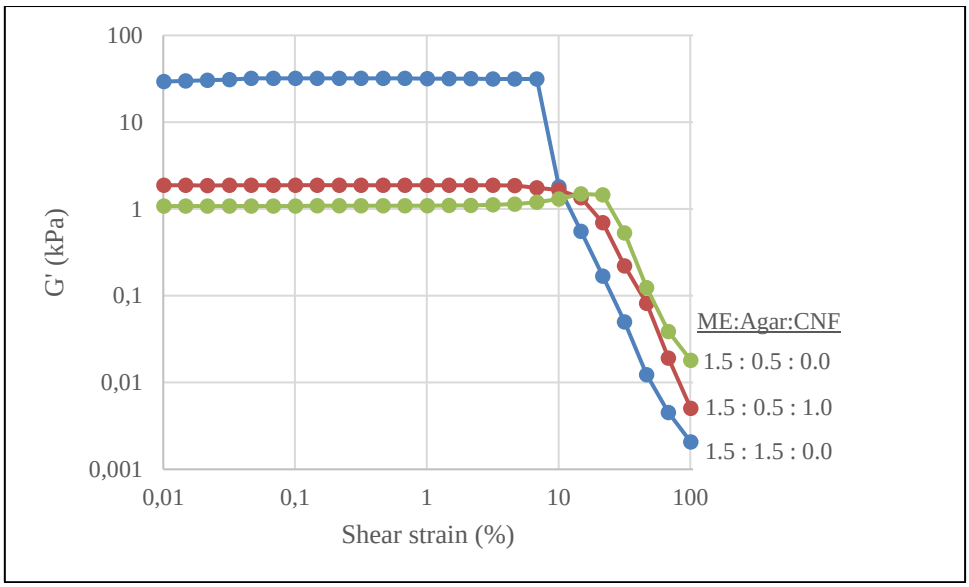

Figure 7: Amplitude sweeps on gels resulting from gelation experiments. Numbers in the legend are weight ratios.

In summary, replacing agar with CNF in the standard MEA resulted in a softer, weaker, but less brittle gel. These changes appeared to have resulted from a change in how the gel structure formed and not just from a dilution in agar concentration relative to the standard MEA.

4. CONCLUSIONS

To our knowledge, this is one of the first studies to examine CNFs in growth media for wood decay fungi. We were unable to completely remove agar in the CNFA growth media because CNF at 1% solids is not a strong or stiff enough gel. However, by reducing the agar to 0.5% we were still able to adequately solidify the media which was conducive for fungal growth and handling. Specific conclusions based on this study are as follows:

- CNFs used as a partial agar replacement did not significantly change growth rates for the eight wood decay fungi examined in this study, apart from *S. lacrymans* which grew faster on CNFA media.
- *G. trabeum* showed significantly higher decay of pine associated with CNFA media.
- Replacing two-thirds of the agar content of a standard MEA solution with CNF greatly altered the formation and structure of the resulting gel. However, understanding the exact nature of these changes would require further research.

- Without the additional 0.5% agar, CNFA remained gel-like and did not harden after heating. To fully replace agar in fungal growth media, combinations of CNF with other gelling agents (e.g., gellan gum, various crosslinkers, etc.) could be explored.
- While the CNF was not as effective of a gelling agent as agar, the rheological performance of the gel formed may be sufficient to serve as growth media.
- Additional studies are likely needed to fully understand the effects of the addition of CNFs on strain virulence over prolonged storage and repeated culturing onto CNF amended agar.

5. ACKNOWLEDGEMENTS

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Supplementary Table 1. Mann-Whitney pairwise analysis (p-values) for the differences in radial growth of white-rot fungi on CNF vs. MEA media.

Day	*T. versicolor*	*I. lacteus*
2	0.050	0.936
4	0.005	0.172
6	0.004	1.000
8	0.008	1.000
12	1.000	1.000

Supplementary Table 2. Mann-Whitney pairwise analysis (p-value) for the differences in radial growth of brown-rot fungi on CNF vs. MEA media.

Day	*G. trabeum*	*R. placenta*	*C. puteana*	*F. radiculosa*	*S. lacrymans*	Day	*W. cocos**
2	0.005	0.010	0.013	0.419	0.627	**1**	0.467
4	0.005	0.005	0.005	0.575	0.092	**2**	0.005
6	0.005	0.010	0.148	0.520	0.078	**3**	0.003
8	0.005	0.005	0.470	0.936	0.031	**4**	1.000
12	0.003	1.000	0.013	0.298	0.020	-	

**W. Cocos* was measured daily to account for its rapid growth rate.

Supplementary Table 3. Mann-Whitney analysis for % mass loss comparisons (p-values).

	CNF vs. MEA		Pine vs. Sweetgum	
Fungus	**Pine**	**Sweetgum**	**CNF**	**MEA**
Wolfiporia cocos	0.01	0.81	0.17	0.81
Rhodonia placenta	0.38	0.81	0.69	0.94
Trametes versicolor	0.93	0.12	0.23	0.40
Irpex lacteus	0.13	0.01	0.01	0.01
Coneophora puteana	0.17	0.94	0.01	0.58
Fibroporia radiculosa	0.93	0.07	0.01	0.01
Serpula lacrymans	0.17	0.69	0.32	0.09
Gloeophyllum trabeum	0.01	0.09	0.94	0.81