In vitro Culture Systems to Test Lignification and Cell Wall Digestibility

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Background

The goal of our GCEP project is to synthesize modified lignins in freshly isolated cell walls and assess their digestibility.

Previously primary cell walls have been isolated from maize cultures, and those walls were modified, analyzed, and assayed for fermentability.

However, most of the lignin in plants is found in the secondary cell wall. Our group is developing culture conditions to generate large enough amounts of secondary cell wall to use as a substrate for modification.

Strategy

Culture cells so they do not make lignin
Isolate non-lignified cell walls
Add monolignols and peroxide back to isolated cell walls
Test digestibility/fermentability of artificially lignified walls

Relationship between gas production (fermentability) and epicatechin hydroxylation.

Nonlignified primary walls isolated from maize cell suspensions were stirred in water and artificially lignified by slowly adding \( \text{H}_2\text{O}_2 \) and lignin precursors CA, SA, and other epicatechin derivatives.

Previous results: Primary cell walls from maize

Why develop a culture system to test modification of secondary cell walls?

Most of the biomass in woody biofuel plants like poplar is in the secondary cell wall. As well, most of the lignin is found in the secondary cell wall. This is where modifications to the cell wall would do the most good.
Secondary cell wall culture systems: tracheary element differentiation

Nodule culture
- Nodules develop large amounts of tracheary elements
- Chief advantage: can induce tracheary element differentiation
- Source: Tobacco leaf cuttings, Poplar stem cuttings
- Source: Arabidopsis root calli

Cell suspensions
- Can induce tracheary element differentiation
- Source: Arabidopsis

TE purification from cultures
- Grind in buffer
- Centrifuge and wash pellet
- or density gradient centrifugation

Making non-lignified cell walls *in vitro*
- Inhibit monolignol biosynthesis in culture
- Block activity early in the phenolpropanoid pathway
- reduce CA and SA pools and thereby reduce lignin.

A. Using chemicals (PAL inhibitor)
B. Mutants in early enzymatic steps (C4H or 4CL)

Arabidopsis mutants available. *e.g.* ref3 alleles with reduced C4H activity in tobacco or poplar, can knock down these activities by RNAi

**ref3 mutants**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Klason lignin, % dry weight</th>
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<tbody>
<tr>
<td>ref3-2</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>ref3-1</td>
<td>9.3 ± 0.2</td>
</tr>
<tr>
<td>ref3-3</td>
<td>16.6 ± 0.3</td>
</tr>
<tr>
<td>Wild Type</td>
<td>16.9 ± 0.8</td>
</tr>
</tbody>
</table>

**Nodule cultures** - developed as a microculture system for horticultural and forestry crops. by Brent McCown, Eric Zeldin, and colleagues at UW-Madison in the 1980s

Nodules are grown in roller bottles, turning at 1 RPM under lights. The tissue is exposed to air and a small amount of nutrient and hormone-containing medium.

**Nodule cultures are scalable** - can grow dozens of bottles for industrial production of material. They have been used to produce taxol, the microtubule inhibiting anti-cancer drug, a natural product of yew trees (*Taxus spp.*). Ellis et al. (1996) Taxol production in nodule cultures of *Taxus*. J. Nat. Prod. 59:246-50

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**Tobacco nodules**

Cross sections of nodules either bright field or stained with berberine hemisulfate which stains lignin and suberin

Conditions for producing nodules with the most secondary wall varies with the plant variety. Most nodules contain 40-50% secondary wall.

We test different amounts of auxin, cytokinin, and sucrose as we continue to optimize conditions. Then we can attempt to grow low-lignin nodules.

These nodules were grown in 0.5 mg/L NAA, 0.2 mg/L BA, 3% sucrose. In two months, 5g nodules >1 mm in diameter grew to >60g nodules.

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**Poplar nodules**

These poplar nodules were grown in 7.5 mg/L NAA, 0.9 mg/L BA, 3% sucrose. In one month, 6g nodules grew to 20g nodules.

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**Arabidopsis cultures do not seem to make nodules**

TE induction from suspension culture with 1 μM Brassinolide to about 30% of cells in culture

TEs are dead cells. Culture must be grown under non-inducing conditions, then BL is added to induce differentiation.