Considerations for Contamination Cleanup in Plant Tissue Culture

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Outline

- Disinfecting the surface of explant tissue
- Indexing to avoid tissue multiplication with contamination
- Worst-Case Scenario
- Strategy
- Molecular Mode of Action and Caveats
  - Antimicrobials
  - Antifungals
  - Antivirals
Explant Surface Disinfection

- Many of the disinfectants are amenable to free-radical formation/oxidation
- Dissolution, adjusting pH will often lead to increased rates of decomposition
- Prepare your solutions fresh!
- Surfactants/Alcohols are used to lower the surface tension of water (e.g. wet the hydrophobic surface of explant tissue)
  - Disinfectant solutions usually contain 2-10 drops of surfactant/L
  - Stay above critical micelle concentration (CMC)
    - Tween 20 CMC = 0.006% (w/w)
  - With increased explant surface area (e.g. larger number of explants to be disinfected) higher concentrations of surfactant needed
    - Some commercial micropropagation labs will pre-wash with surfactant (or even soaps), alcohols prior to disinfection
**Explant Surface Disinfectants**

- **Hypochlorous Acid (HOCl)**
  - Oxidizes Protein Thiols (-SH) & Halogenates any Protein Free amines → Enzyme dysfunction (Summers et al. 2012) & Unfolding (Winter et al. 2008)
  - 10% Bleach (0.5-0.83% NaOCl) = expose 5-30 min
    - Alkaline solution (pH 10)
    - H-OCl, pKₐ = 7.5
      - @ pH 5.5, 99% is HOCl, 1% is -OCl
  - NaDCC (2-5 g/L) – less phytotoxicity because of neutral pH at dissolution, and less need to rinse
    - Forms HOCl in aqueous solution

- **Chlorine dioxide (ClO₂)**
  - Chlorite (ClO₂⁻) most common species in aqueous solution above pH 3
  - 0.001-0.01% used to disinfect apple (Kreske et al. 2006)
  - 0.0025% used in place of autoclaving (Cardoso 2012)

- **Hydrogen peroxide (H₂O₂)**
  - Stable at pH 3-4 (Solvay Interox 1998)
  - Mixed with acetic acid it forms peracetic acid – very high redox potential

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Winter et al. (2008) Cell Vol. 135 pg. 691-701
Kreske et al. (2006) J. Food Prot. Vol. 69(8) pg. 1892-1903
Indexing to Avoid Tissue Multiplication w/ Contamination

- Consider the tissue type
- Co-culture tissue & microorganism
  - Leifert & Waites Medium (Leifert and Waites, 1989) [L476]
  - ½ MS + Peptone + YE (Reed et al. 2004)
- Streak Tissue on plate to check for growth
  - Nutrient Agar/Broth [N601/N611]
  - Bacterial Medium 523 [B129]
  - Sabouraud Dextrose Medium [S7536]
  - Potato Dextrose agar/broth-fungi [P772/P762]
  - Czapek Dox Broth-fungi [C506/C443]

Indexing to Avoid Tissue Multiplication w/ Contamination

- Endophytes- bacteria and fungi in vascular tissues and in intercellular spaces and intracellular compartments
  - In Banana, bacteria was found between the cell wall & plasma membrane (Thomas and Chandra Sekhar, 2014)
- It is important to re-index (especially if you are changing the plant tissue culture medium-type!!!) as it is common for microorganisms to overcome:
  - Media pH (pH 4 is bacteriostatic to Pseudomonas & Bacillus (Leifert and Waites, 1992)
  - Media salt concentration
  - Phenolics excreted by the tissue (banana, sugar cane)
  - Plant Growth Regulators have affected yeast growth

Worst-Case Scenario

- 100 explants are contaminated
- You have been using bacteriostatic agents, and now are trying to get your tissue off of it
- Gram-staining your micro-organism showed both gram+ & gram-
- There isn’t time/money to repeat the disinfection of new explant tissue for multiplication at the stage you are currently at....

What do you do?
Strategy

- Go to the Literature to see what has been used for your specific Plant species
- Bactericidal over Bacteriostatic
  - Eliminate – do not just suppress the growth of bacteria
- Gram Stain
  - Address your contamination with the proper Gram(+) or Gram(-) antimicrobial
- 16S ribosomal DNA sequencing is becoming standard for microbe identification (5-7 days)
  - PCR amplify and sequence first 500-600 bp
  - Compare to gene library of ~2000 species
- Combine antibiotics so that they can kill micro-organism in different ways
  - Inhibit bacterial protein synthesis and cell wall synthesis
  - Some antibiotic combinations can be phytotoxic
- Test multiple concentrations to determine the proper dose
  - Dose-response curve
**Bactericidal**
- Aminoglycosides
- Cephalosporins
- Penicillins
- Glycopeptides
- Rifampicin

**Bacteriostatic**
- Chloramphenicols
- Tetracyclines
- Macrolides (Erythromycin)

<table>
<thead>
<tr>
<th>Gram (-)</th>
<th>Gram (+)</th>
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<tbody>
<tr>
<td>Aminoglycosides: some Gram (+) activity</td>
<td></td>
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<tr>
<td>Cefotaxime: 3\textsuperscript{rd} Generation Cephalosporins some Gram (+) activity</td>
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<tr>
<td>Penicillins (e.g. Carbenicillin, Ticarcillin active ingredient in Timentin, Ampicillin, Amoxicillin)</td>
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<tr>
<td>Rifampicin: some Gram (+) activity</td>
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<tr>
<td>-</td>
<td>Glycopeptides (Vancomycin, Bacitracin Zinc, Polymyxin B)</td>
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Inhibiting Protein Synthesis (PS)

- **Aminoglycosides**
  - Amino-linked sugars
  - Bind to 30S or 50S subunits on the ribosome, blocking translation of mRNA to the growing peptide
  - “mycins”

- **Indirectly Inhibits PS**
  - Rifampicin blocks nucleotide synthesis by binding to an RNA polymerase subunit

Caveats to Inhibiting Protein Synthesis (PS)

- Chloroplasts & Mitochondria contain 70S ribosomes!
  - Aminoglycosides are known to inhibit cell proliferation and differentiation during transformation regeneration
  - Leaf explant tissue is generally affected across all species, but different species can be differentially sensitive to various aminoglycosides (Padilla and Burgos, 2010)
  - Chloroplast PS is required for normal plant development in tobacco (Ahlert et al. 2003).
- Rifampicin has been shown to completely inhibit RNA polymerase in chloroplasts from C. reinhardtii at 100 μg/mL (Surzycki 1969). Yet no inhibition was seen below 50 μg/mL.

Ahlert, Ruf, and Bock. (2003) PNAS Vol. 100 (26) pg 15730-15735
Surzycki. (1969 ) PNAS Vol. 63 (4) pg. 1327-1334
Inhibiting Bacterial Cell Wall Synthesis

- β-Lactam rings are broken and covalently attached to a serine essential for peptide cross-linking on Penicillin-Binding Proteins (PBP).
- "cillins", cefotaxime
- Glycopeptides (GP) achieve the same end result but through blocking the peptides to be cross-linked

β-lactam Solution Stability w/ FTIR

Absorbance decreases when β-lactams react with Penicillinase

υ(C=O) = 1755 cm⁻¹

Incubated 50mg Carbenicillin with 500,000 IU Penase (BD Difco) for 3hr at 37°C
**β-lactam Solution Stability w/ FTIR**

- **Intactβ-lactam rings $\nu_{(C=O)}$ absorb at 1750-1780 cm$^{-1}$ in FTIR (Fourier Transform Infrared) spectrums.**

- **Lambert-Beer’s Law for Attenuated Total Reflectance (ATR)**
  
  \[ A = N \varepsilon bC \]

  - $N = 1$ (specified by ATR type)
  - $b = 2 \mu$m (specified by ATR type)
  - $\varepsilon$(Carb) = 0.00006 [mL/mg·μm]

- We are working to validate against potency [μg/mg] to reduce the amount of time the biological assay takes.
Antifungals

- Amphotericin B & Nystatin binds sterols (specifically ergosterol) in plasma membranes, which is the primary mechanism of cell death in yeast (Gray et al. 2012).
- Plant cells have a vast array of sterols
- Fungi sterol content is mostly ergosterol
- Plants are susceptible to the same type of cell death

Antifungals (cont.)

- Carbendazim
  - Inhibit microtubule assembly ($EC_{50} = 4 \mu M$) in yeast and growth ($EC_{50} = 30 \mu M$) (Davidse, 1986)
  - Autoclavable

Antivirals

- Ribavirin (Virazole):
  - Adenosine/Guanosine analogue
  - Extremely broad antiviral activity, inhibiting RNA virus replication
  - It's inhibition of DNA virus activity is not completely understood
  - Eliminated Potato Viruses X, Y, S and M in Potato (Cassells and Long, 1982)
  - Titers of Cucumber Mosaic (CMV) and Alfalfa Mosaic Viruses in plant tissue were reduced significantly when ribavirin 50-100 mg/L was added to the culture medium (Simpkins et al. 1981)

Summary

- Develop robust protocols for explant disinfection
- Index your explants post-disinfection
- If you need to use antibiotics/antifungals
  - Bacteriocidal to eliminate in a single dose
  - Consider the mechanism of action
  - Test various concentrations of the antibiotic/antifungal with your explant
- Be watchful of media changes in your culture...there are endophytes lurking
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Questions?

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