Considerations for Contamination Cleanup in Plant Tissue Culture

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Outline

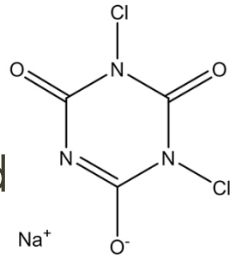
- Disinfecting the surface of explant tissue
- Indexing to avoid tissue multiplication with contamination
- Worst-Case Scenario
- Strategy
- Molecular Mode of Action and Caveats
  - Antimicrobials
  - Antifungals
  - Antivirals

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 , pK_A = 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

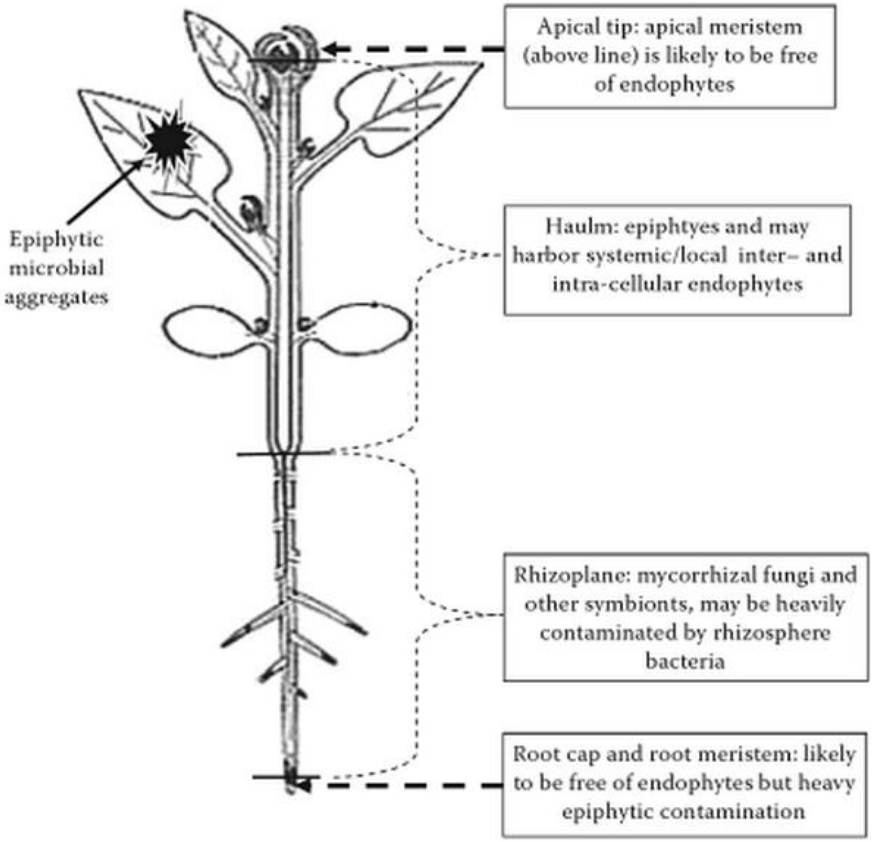


- Chlorine dioxide (ClO_2)
  - Chlorite (ClO_2^-) most common species in aqueous solution above pH 3
  - 0.001-0.01% used to disinfect apple (Kreske *et al.* 2006)
  - 0.0025% used in place of autoclaving (Cardoso 2012)
    - Hydrogen peroxide (H_2O_2)
      - Stable at pH 3-4 (Solvay Interox 1998)
      - Mixed with acetic acid it forms peracetic acid – very high redox potential

Summers *et al.* (2012) *Biochem. Biophys. Res. Comm.* Vol. 425 pg. 159-161.
Winter *et al.* (2008) *Cell* Vol. 135 pg. 691-701
Cardoso and Teixeira de Silva (2012) *In Vitro Cell Dev. Bio. - Plant* Vol. 48 pg 362-368
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]



Cassells. (2011) in *Plant Tissue Culture, Development and Biotechnology* Ed: Trigiano & Gray. CRC Press pg. 223-238.

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

Thomas and Chandra Sekhar (2014) *AoB Plants.* doi: 10.1093/aobpla/plu002
Leifert & Waites (1992) *J. App. Microbiol.* Vol. 72 pg. 460

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

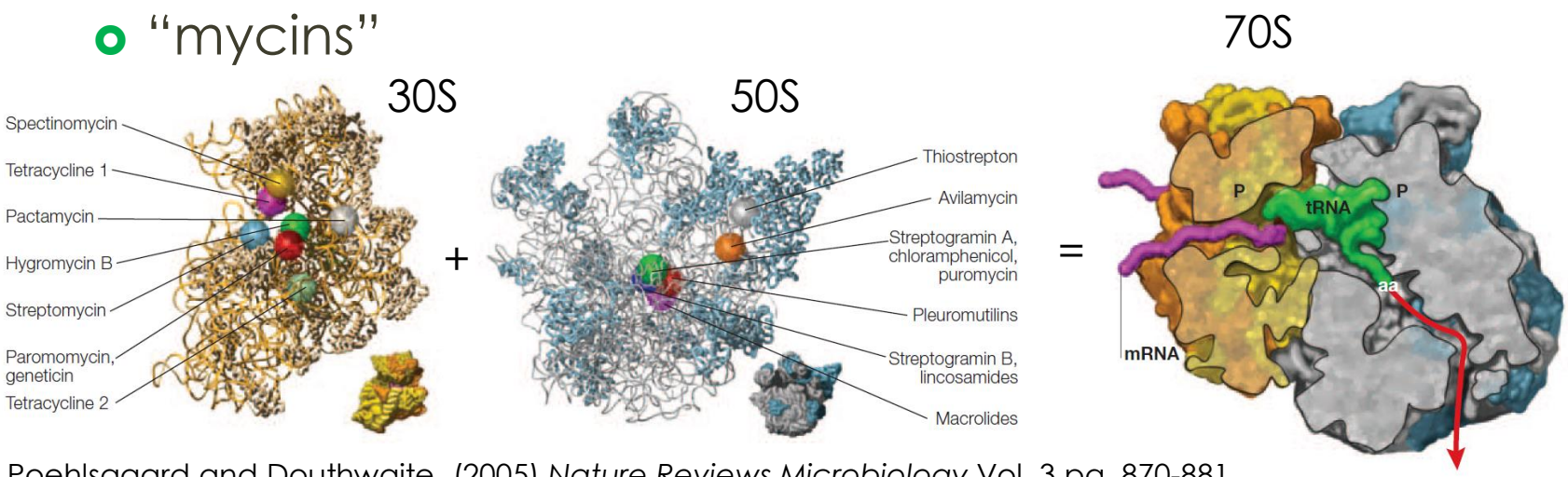
Bacteriostatic

- Chloramphenicols
- Tetracyclines
- Macrolides (Erythromycin)

Gram (-)	Gram (+)
Aminoglycosides: some Gram (+) activity	
Cefotaxime: 3rd Generation Cephalosporins some Gram (+) activity	
Penicillins (e.g. Carbenicllin, Ticarcillin active ingredient in Timentin, Ampicillin, Amoxicillin)	
Rifampicin: some Gram (+) activity	
-	Glycopeptides (Vancomycin, Bacitracin Zinc, Polymyxin B)

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



Poehlsgaard and Douthwaite. (2005) *Nature Reviews Microbiology* Vol. 3 pg. 870-881

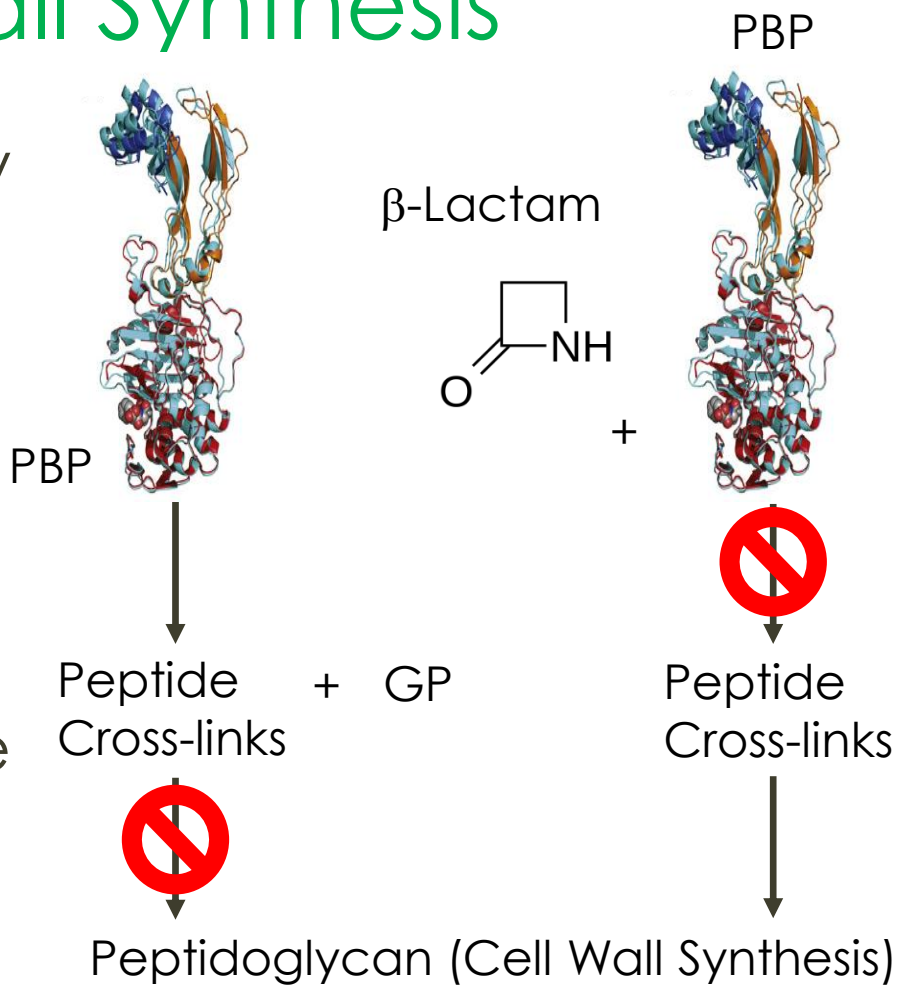
Caveats to Inhibiting Protein Synthesis (PS)

- 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.

Padilla and Burgos (2010) Plant Cell Reports Vol. 29 pg. 1203-1213
Ahlert, Ruf, and Bock. (2003) *PNAS* Vol. 100 (26) pg 15730-15735
Surzycki. (1969) *PNAS* Vol. 63 (4) pg. 1327-1334

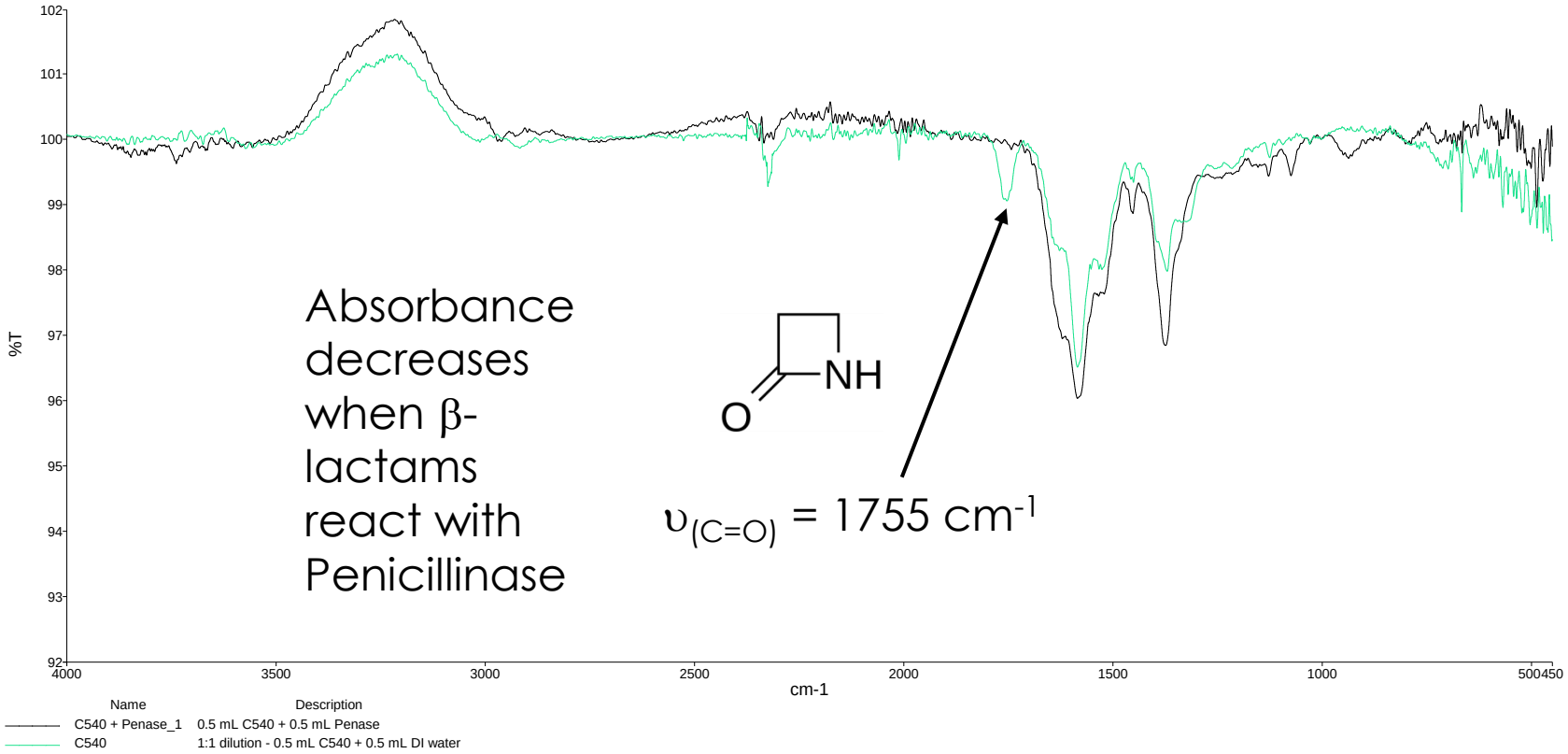
Inhibiting Bacterial Cell Wall Synthesis

- 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



Sainsbury *et al.* (2011) *Journal of Molecular Biology* Vol. 405 pg. 173-184

β-lactam Solution Stability w/ FTIR



Absorbance decreases when β-lactams react with Penicillinase

$\upsilon_{(C=O)}$ = 1755 cm^{-1}

Incubated 50mg Carbenicillin with 500,000 IU Penase (BD Difco) for 3hr at 37°C

β-lactam Solution Stability w/ FTIR

- **Intact** β-Lactam rings $\upsilon_{(C=O)}$ absorb at 1750-1780 cm^{-1} in FTIR (Fourier Transform Infrared) spectrums
- Lambert-Beer's Law for Attenuated Total Reflectance (ATR)

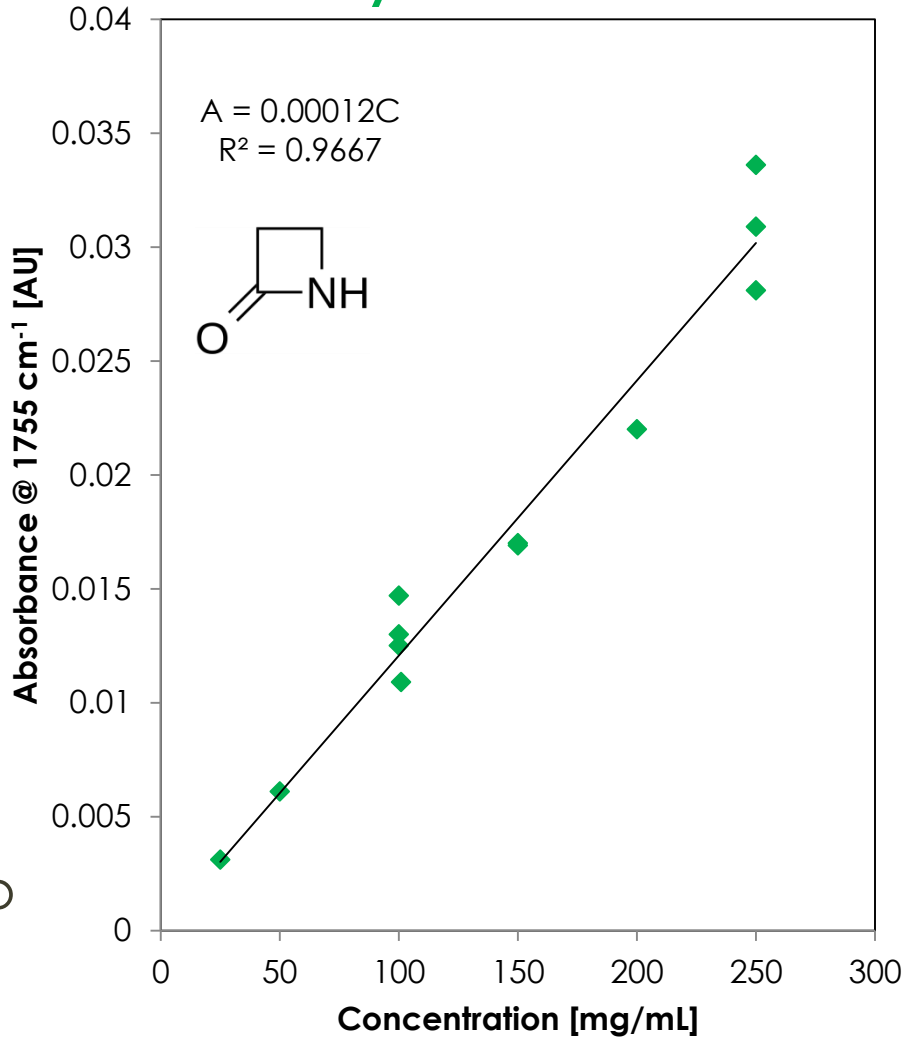
$$A = N\varepsilon bC$$

N = 1 (specified by ATR type)

b = 2 μm (specified by ATR type)

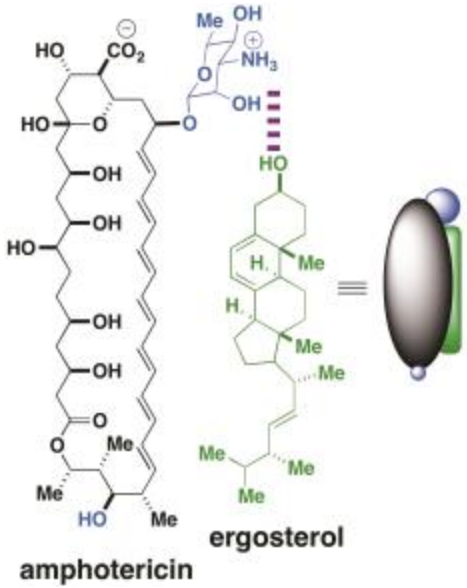
$$\varepsilon(\text{Carb}) = 0.00006 \left[\frac{mL}{mg \cdot \mu m}\right]$$

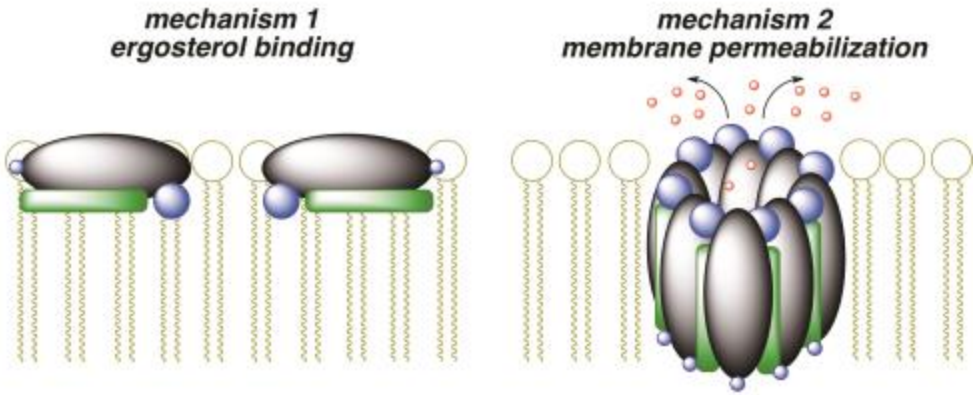
- We are working to validate against potency [μg/mg] to reduce the amount of time the biological assay takes



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 an vast array of sterols
  - Fungi sterol content is mostly ergosterol
- Plants are susceptible to the same type of cell death

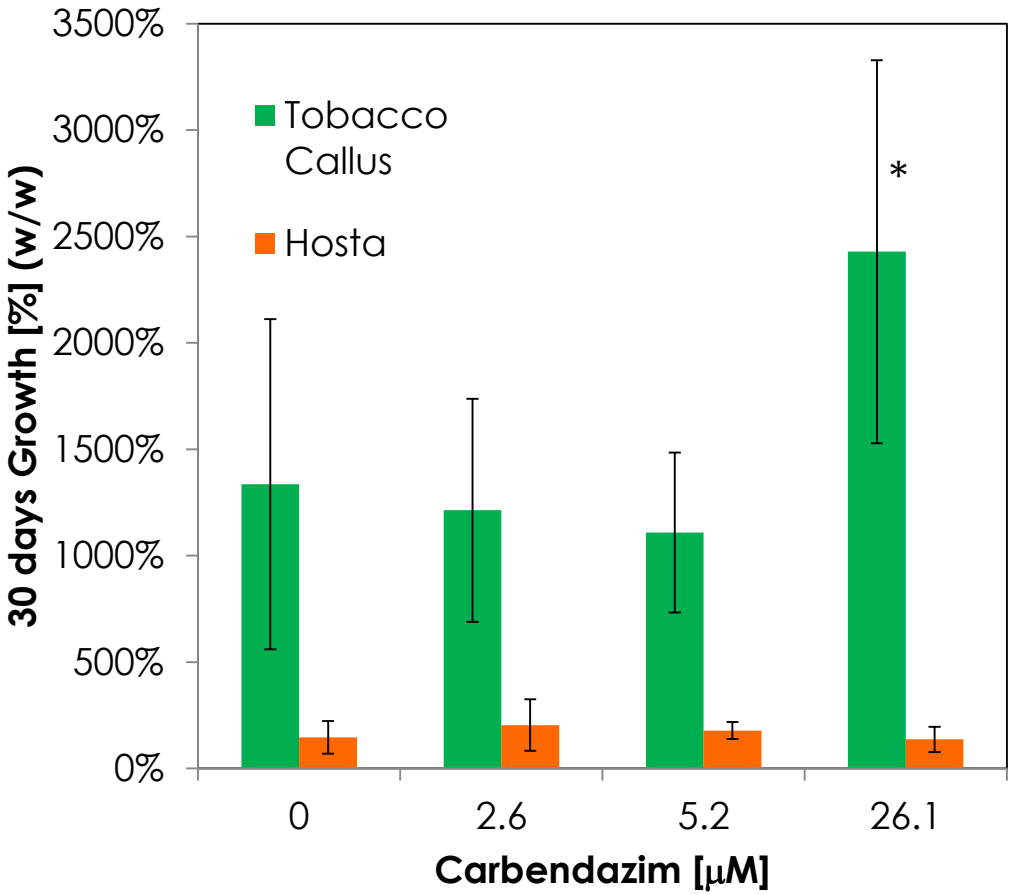




Gray *et al.* (2012) *PNAS* Vol. 109(7) pg. 2234-2239.

Antifungals (cont.)

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



* p = 0.001

Davidse (1986) *Ann. Rev. Phytopathol.* Vol. 24 pg. 43-65.

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 the culture medium (Simpkins *et al.* 1981)

Cassells and Long (1982) *Potato Research* Vol. 25(2) pg. 165-173.
Simpkins *et al.* (1981) Ann. App. Biology Vol. 99(2) pg 161-169.

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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