Rapid Diagnostic Test for Pitch Canker

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Presentation outline

- Background on pitch canker
- The problem
- Seed screening project
  - ISTA culture based method
  - Intro to PCR
  - New PCR based seed screening method
  - Current status
  - Potential applications

Background Information

- Pitch canker is a serious disease of pines
- **Causal agent:** *Fusarium circinatum*
  - (previously known as *Fusarium subglutinans* f. sp. *pini* or *Fusarium moniliforme* var. *subglutinans*)
- **Host range:**
  - Conifers, mostly *Pinus* spp.
- **Symptoms:**
  - death of shoots, occasionally trees
  - crooked stems and forks
  - pitch bleeding from shoots, resin-soaked wood
  - Can infect at any stage in pine development
  - Pre- and post-emergence "damping off"
  - Infected seedlings are often planted
- **Signs:**
  - rarely salmon colored sporodochia
  - microconidia (oval) and macroconidia (canoe-shaped)

Sporodochia

Photo by G.M. Blakeslee from http://www.fl-dof.com/publications/fh_pdfs/Pitch%20Canker%20of%20Southern%20Pines.pdf

Pitch canker disease cycle

Nitrogen can increase disease

Seeds and cones get infected

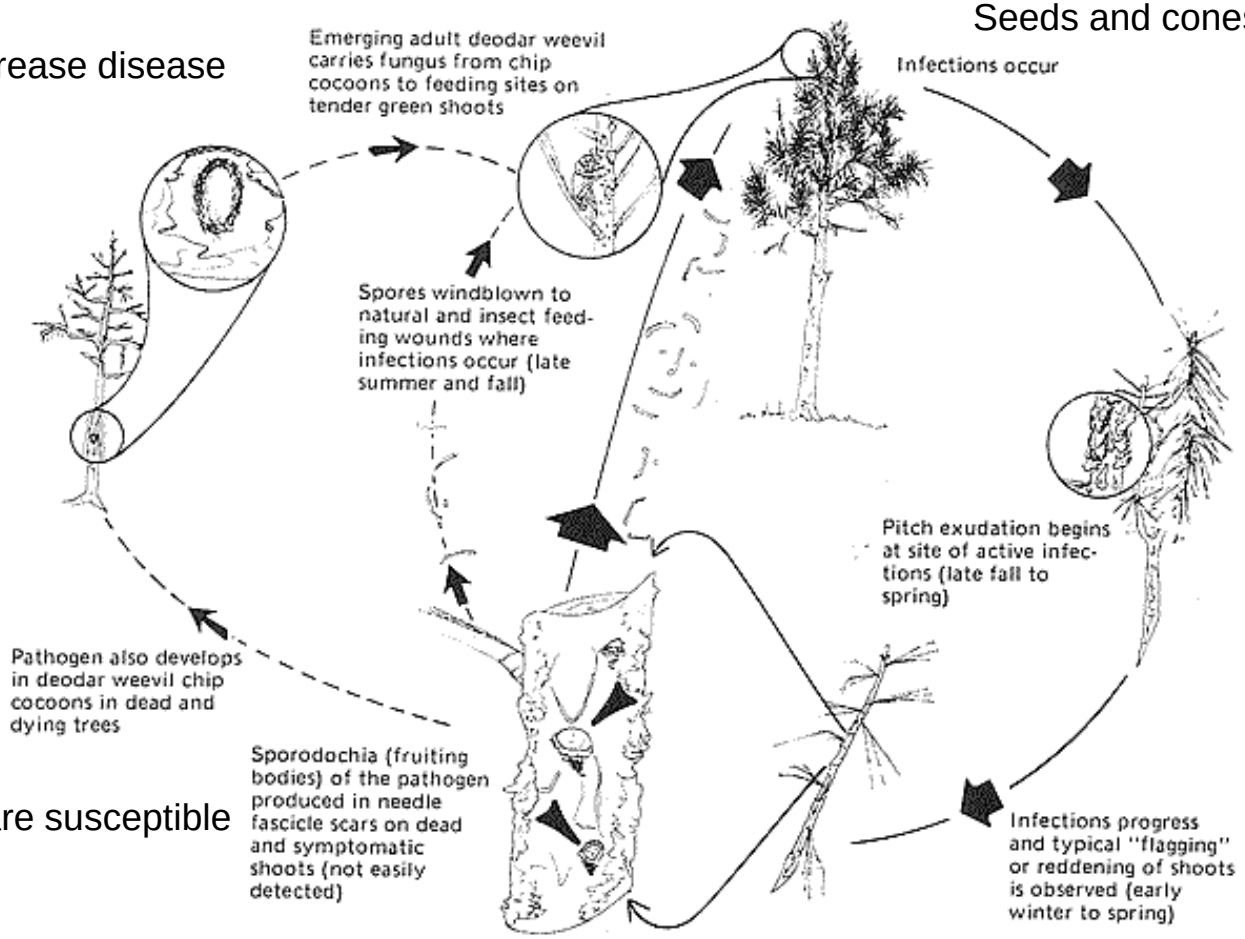


Fig. 13. Simplified life cycle of the pitch canker fungus on slash pines in Florida.

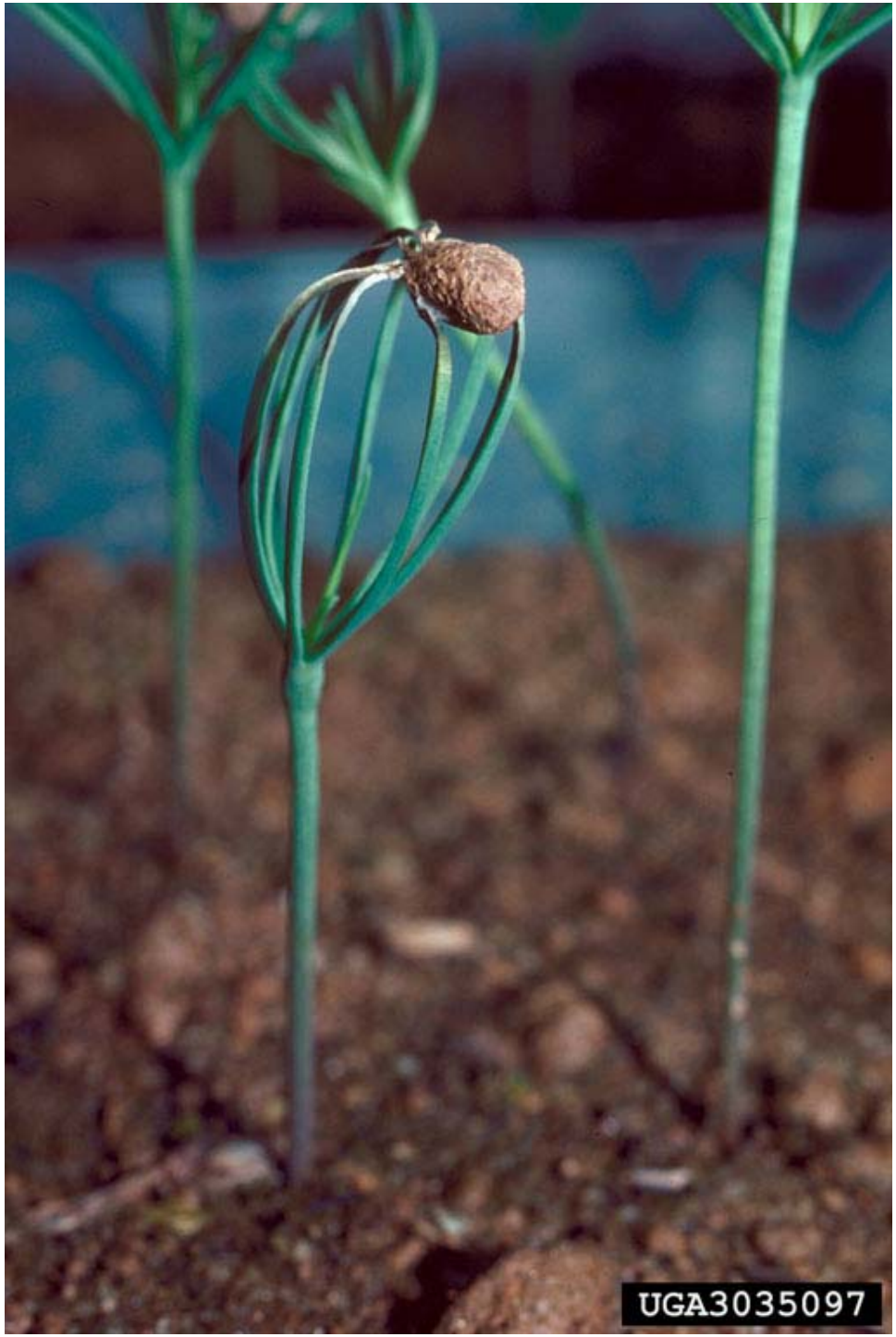
Pines of any age are susceptible

http://www.fl-dof.com/publications/Insects_and_Diseases/td_cs_pitch_canker_fig13.html


UGA1501012

Canker margin

Cotyledon infection from contaminated seed



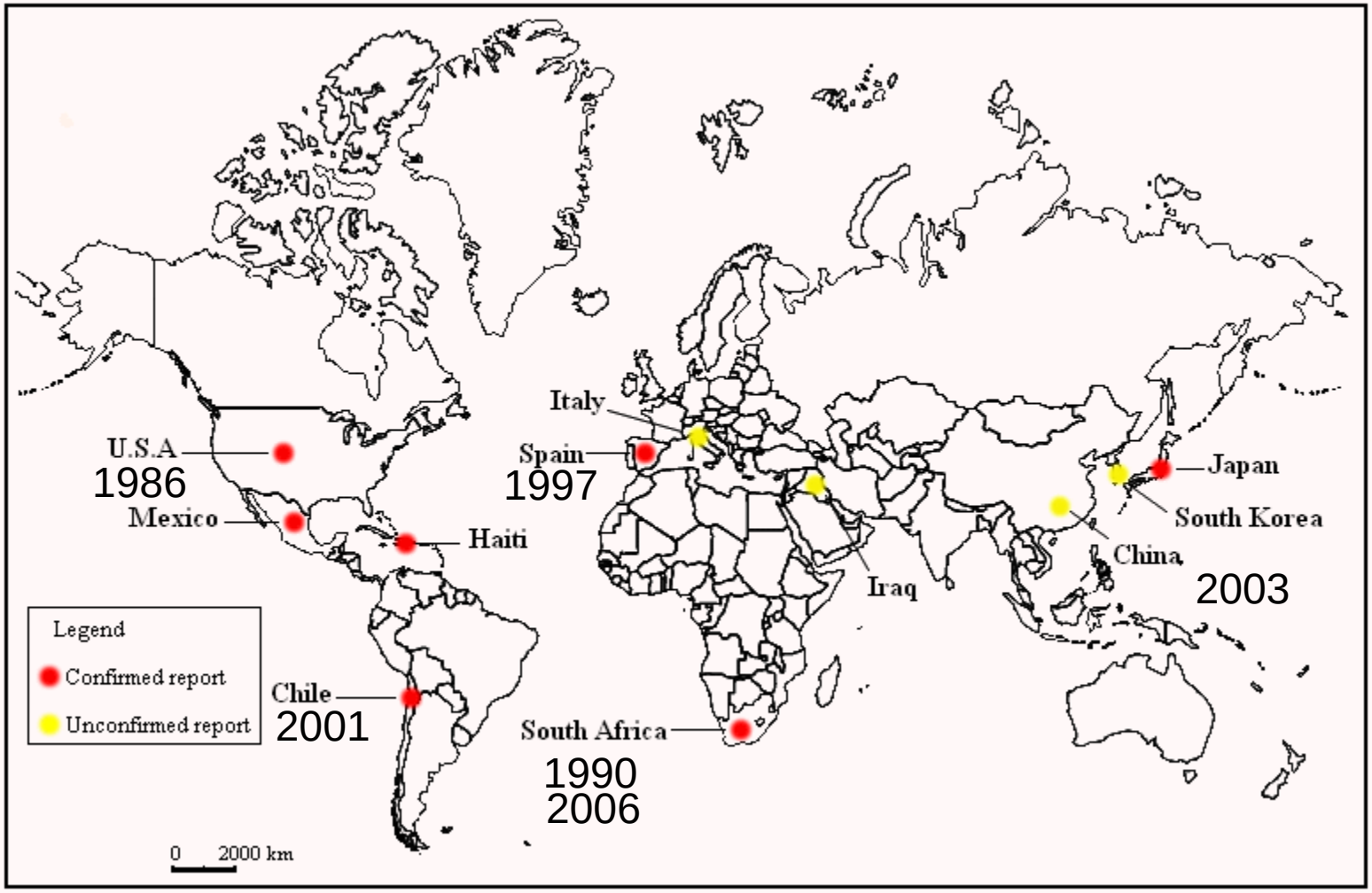


Figure 1. Worldwide distribution of pitch canker (Ganley, 2006).

Why is regulation needed

- Massive plantations exist of susceptible pine species such as *P. radiata* and *P. taeda* in many countries
- Pitch canker does not currently exist in many areas
  - Need to keep new strains out
- Seeds are being shipped globally
- Pitch canker can be transmitted
  - Contamination of the seed coat
  - Contamination of the embryo

The problem……

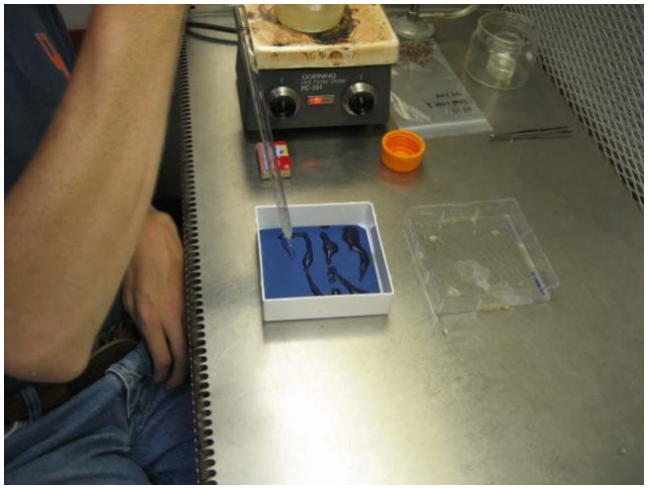
- Currently, seeds are tested and certified using the International Seed Testing Association Guidelines
- ISTA Guidelines require testing 400 seeds per seedlot
- Seeds are tested by a "blotter paper method" that involves growing *F. circinatum* out of crushed seeds onto blotter paper
- This process is very slow, laborious, and prone to false negatives

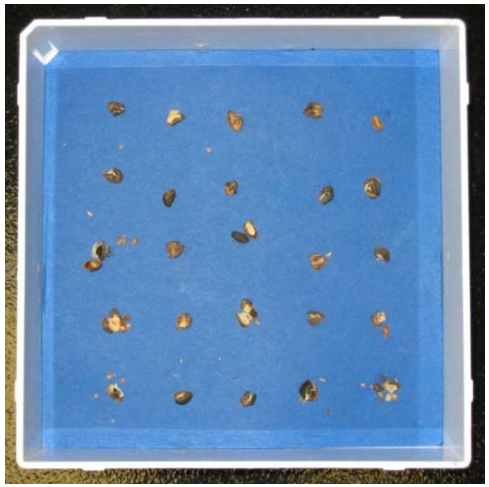
Objectives of study

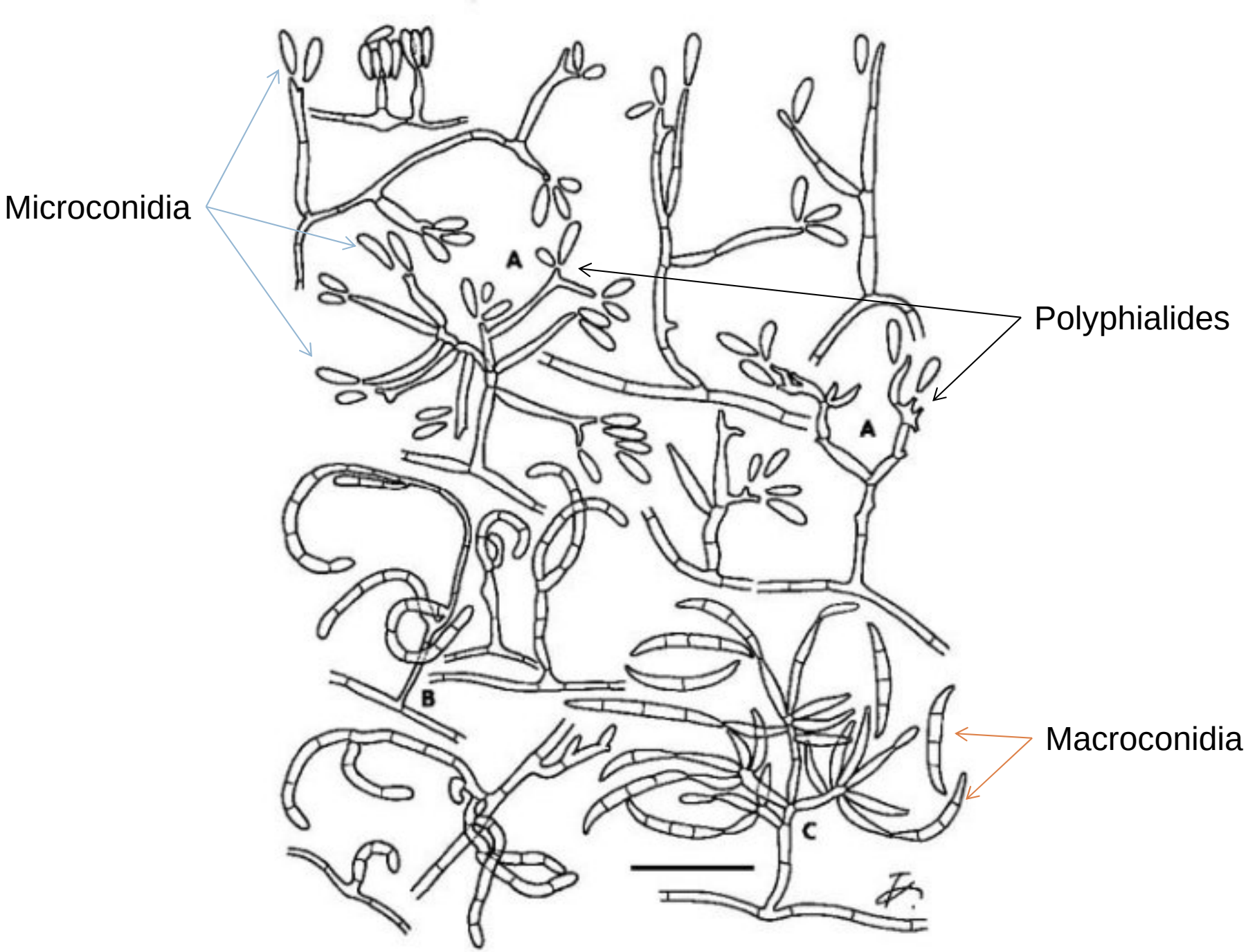
- Develop qPCR seed screening method
- Compare qPCR method to current culture-based method by screening 6 slash pine seed lots using both methods
  - Compare detection results
  - Compare time and cost associated with both methods

ISTA culture based screening method

- Based on
  - Anderson, R.L. 1986. New method for assessing contamination of slash and loblolly pine seeds by *Fusarium moniliforme* var. *subglutinans*. Plant Disease 70: 452-453.
- Relies on placing seeds on selective media and identifying fungi that grow based on morphology under a microscope
- Also called blotter paper method

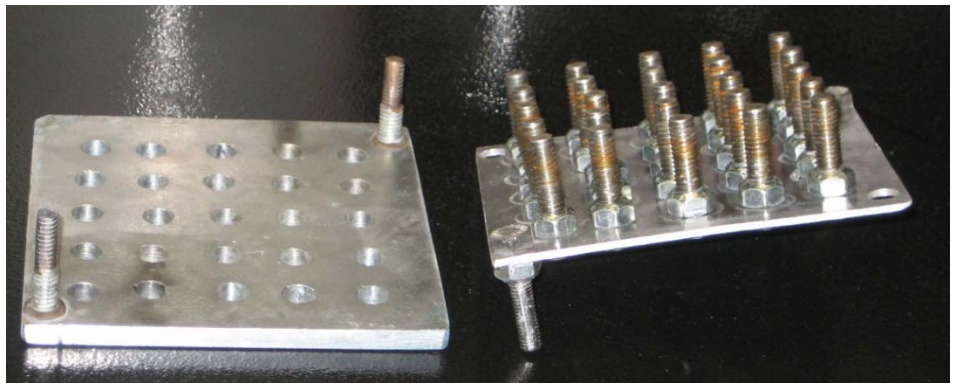


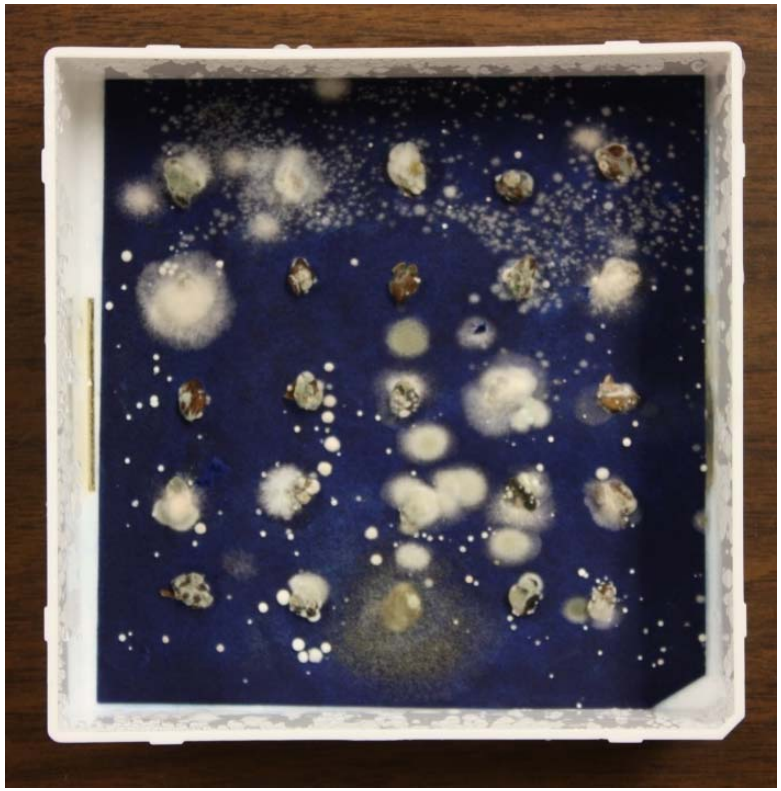



Microconidia
Polyphialides
Macroconidia
A
A
B
C

Culture based screening method

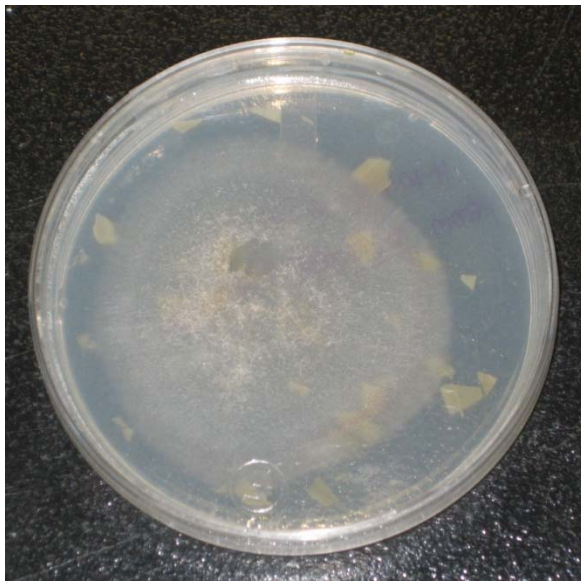
- Crush seeds on blotter paper with selective media

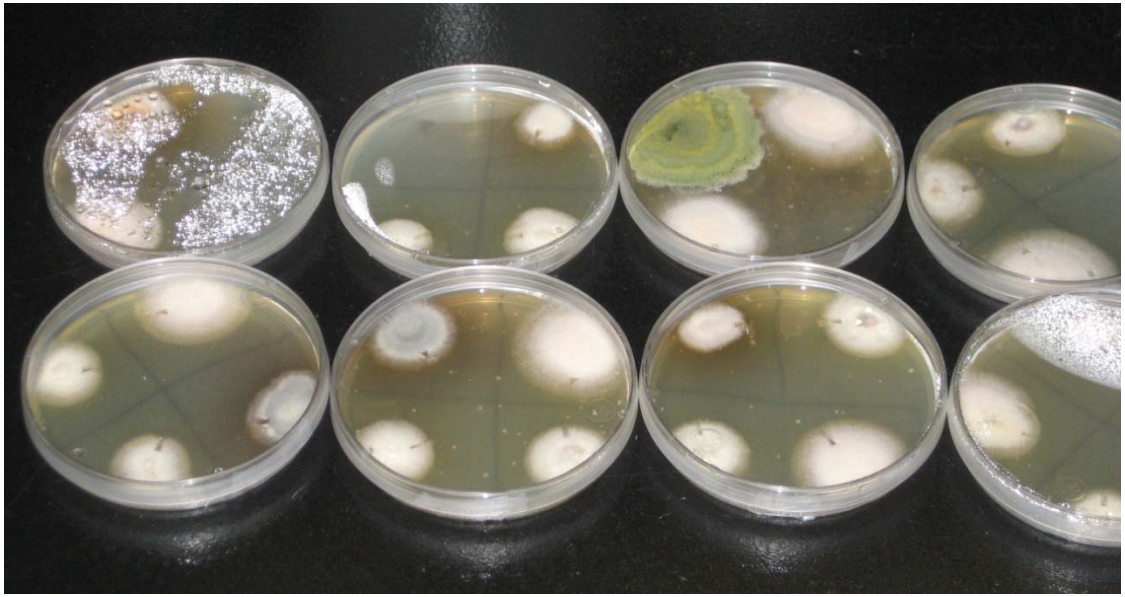




Culture based screening method

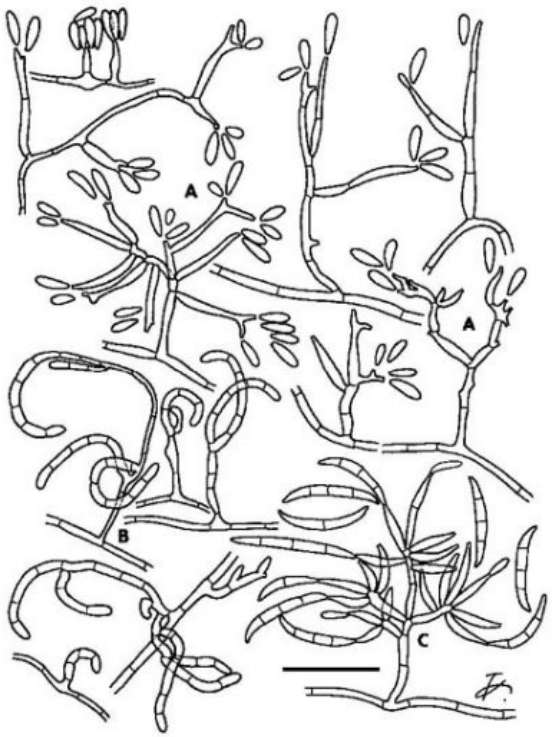
- Subculture on carnation water agar
  - Contamination





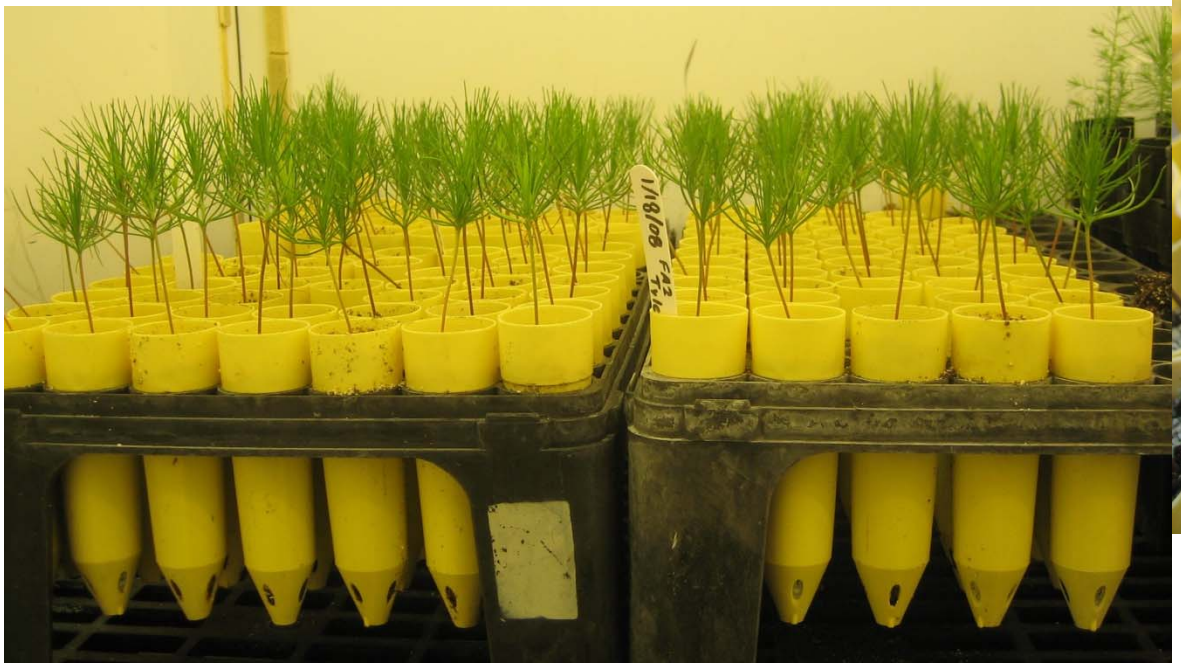
Culture based screening method

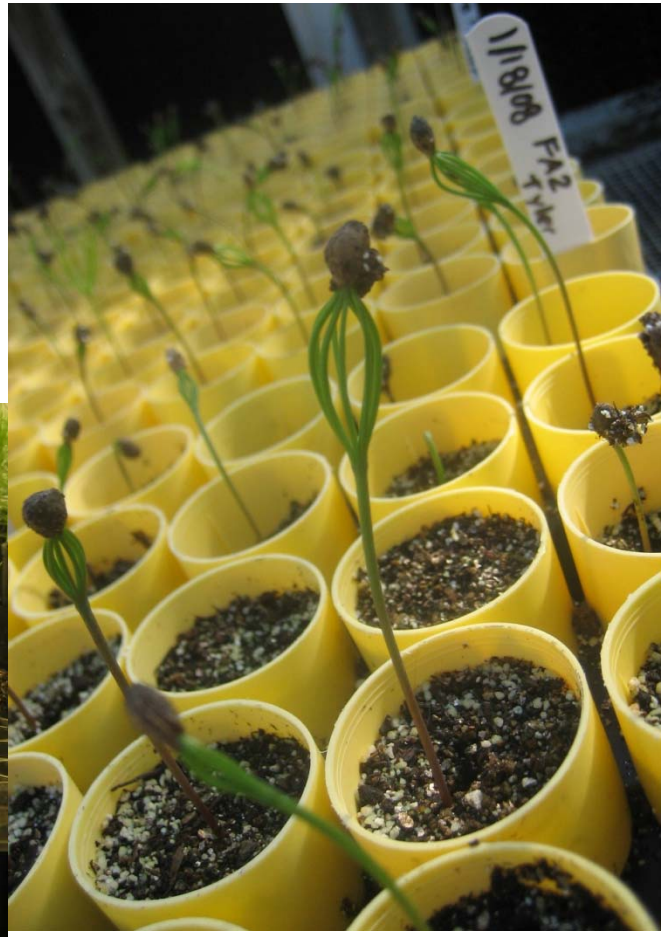
- Identified morphologically under a microscope
  - 3-7 hours



Culture based screening method

- Inoculate seedlings to prove pathogenicity





What is PCR?

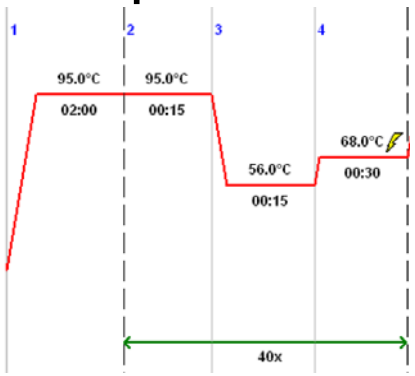
- Polymerase Chain Reaction (PCR)
- Method of replicating DNA without living organisms
- Amplifies a specific region of DNA (can be used to identify species)
- Driven by change in heat
- Prior knowledge of organism's DNA is needed
- Can be used to detect organism presence in samples

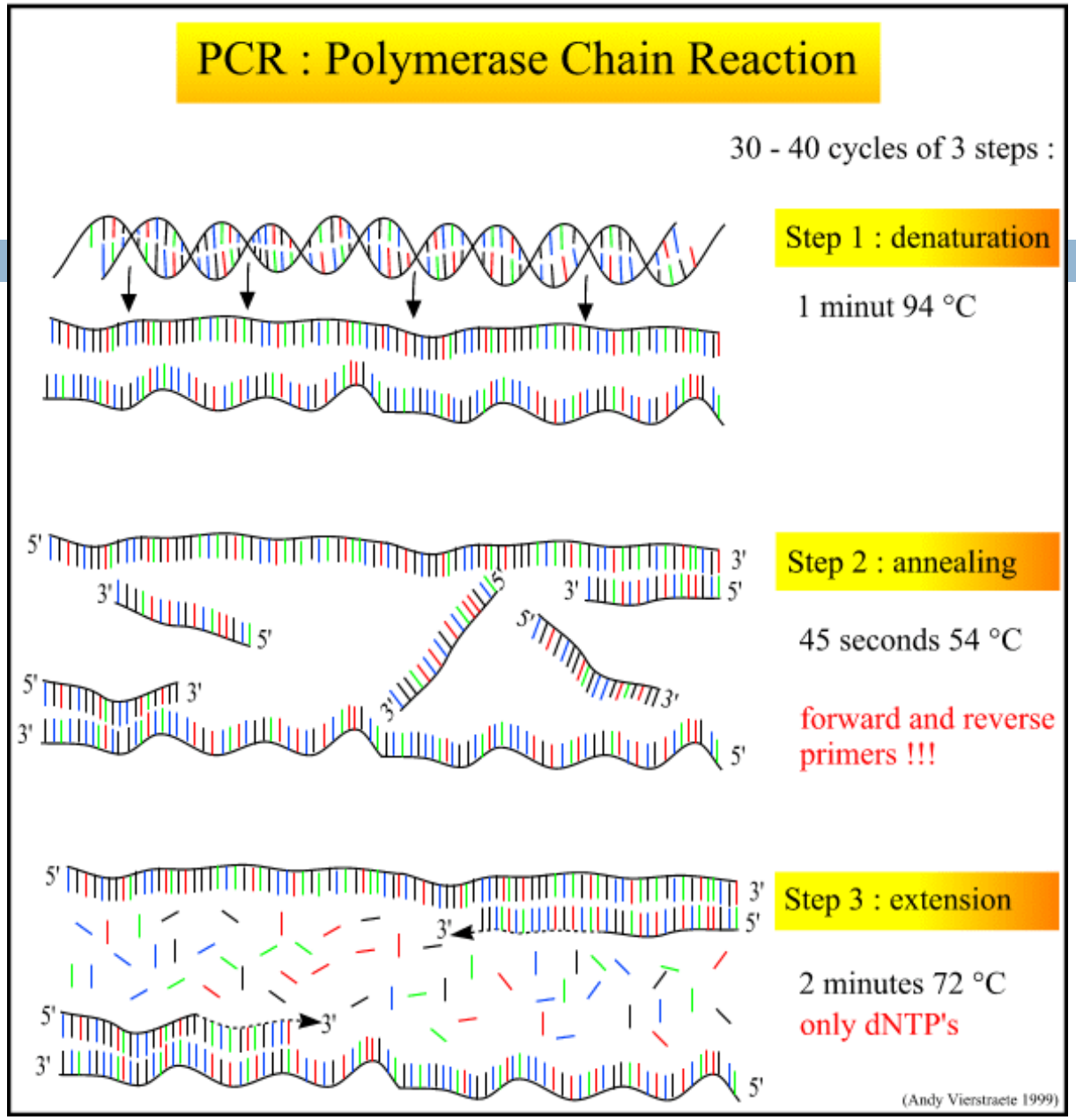
PCR components

- Template DNA- DNA from sample to be replicated
- Primers- single stranded DNA that specify the end points of the PCR product (provide specificity)
- *Taq* polymerase- adds single nucleotides to the ends of the primers and makes a new DNA strand
- Free nucleotides- A, T, G, C building blocks of DNA required to make replicate strands

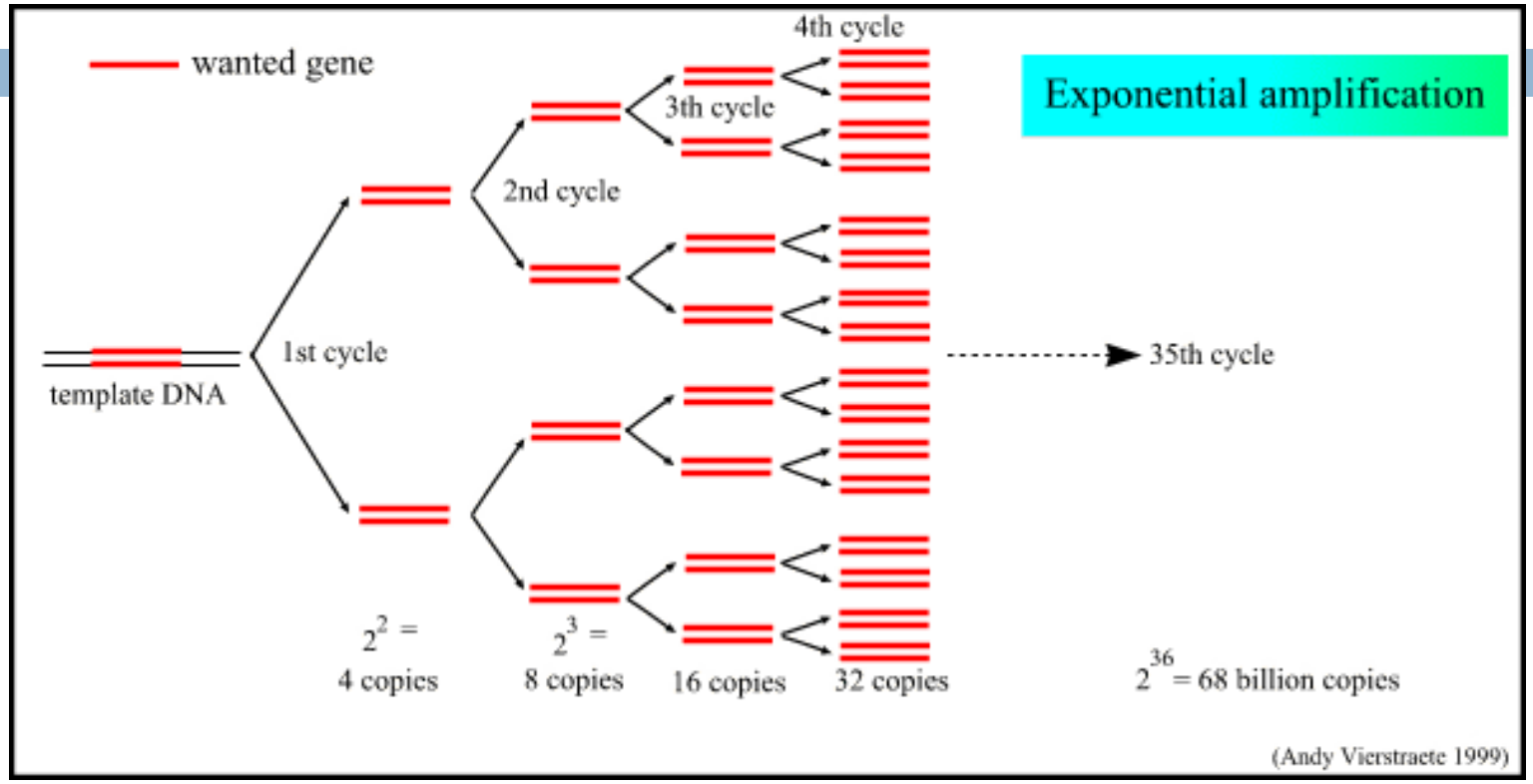
PCR steps

- Denaturation- separation of double-stranded DNA to single strands
- Primer annealing- primers bind to a specific location on the single stranded DNA
- Extension- *Taq* polymerase, an enzyme, adds single nucleotides to the ends of the primers
- These steps are repeated 30 to 40 times. After each cycle the number of copies of the DNA doubles.





http://users.ugent.be/~avierstr/principles/pcr.html



http://users.ugent.be/~avierstr/principles/pcr.html

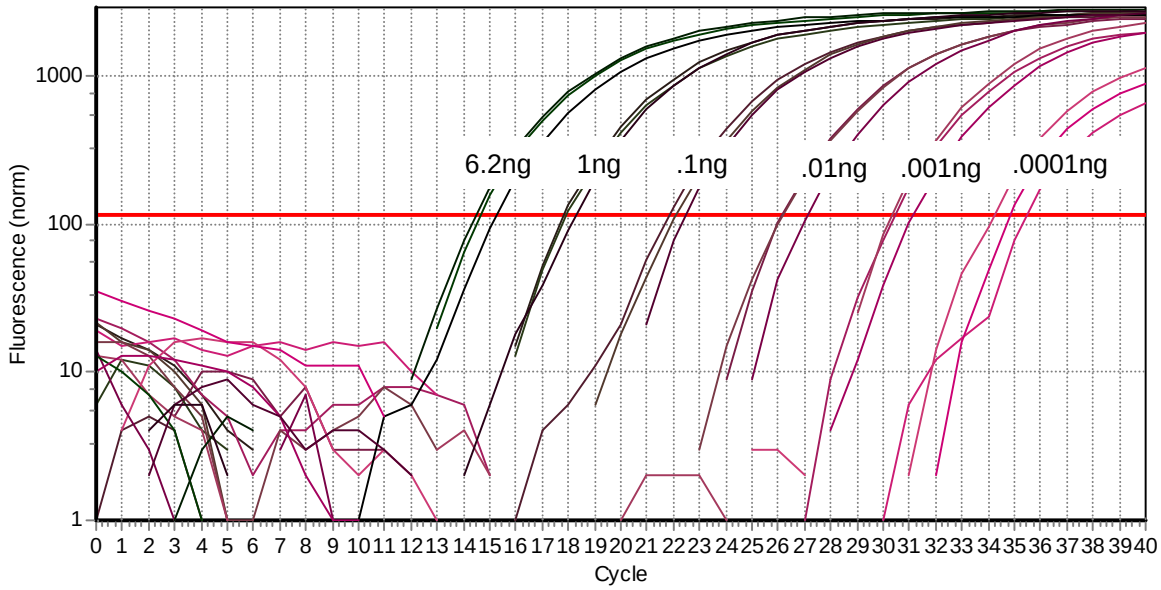
Real time PCR, qPCR

- Quantifies the initial amount of the DNA template
  - highly specific, sensitive, and reproducible
- Similar to regular PCR but the amplification is read by a computer in real-time
- Faster and more sensitive that traditional PCR
- Commonly used to test for presence of pathogens
  - Examples: Sudden oak death, soybean rust, bird flu

qPCR

- Relies on the change in fluorescence due to amplification of DNA
  - Generally as amplification increases, fluorescence increases
  - Fluorescence is measured after each cycle
  - The more amplified DNA in the PCR reaction the less number of cycles needed for the fluorescence to significantly increase over the background.

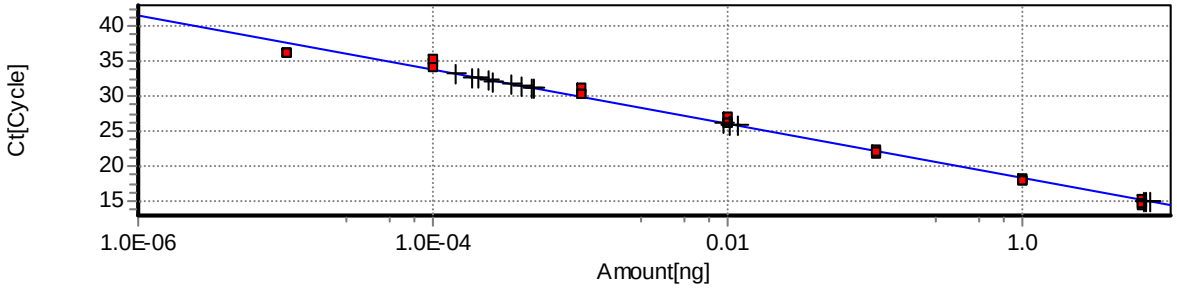
qPCR



Cycle threshold (Ct)

Threshold: 117 (Noiseband)

Baseline settings: automatic, Drift correction OFF



Slope: -3.883

Y-Intercept: 18.32

Efficiency: 0.81

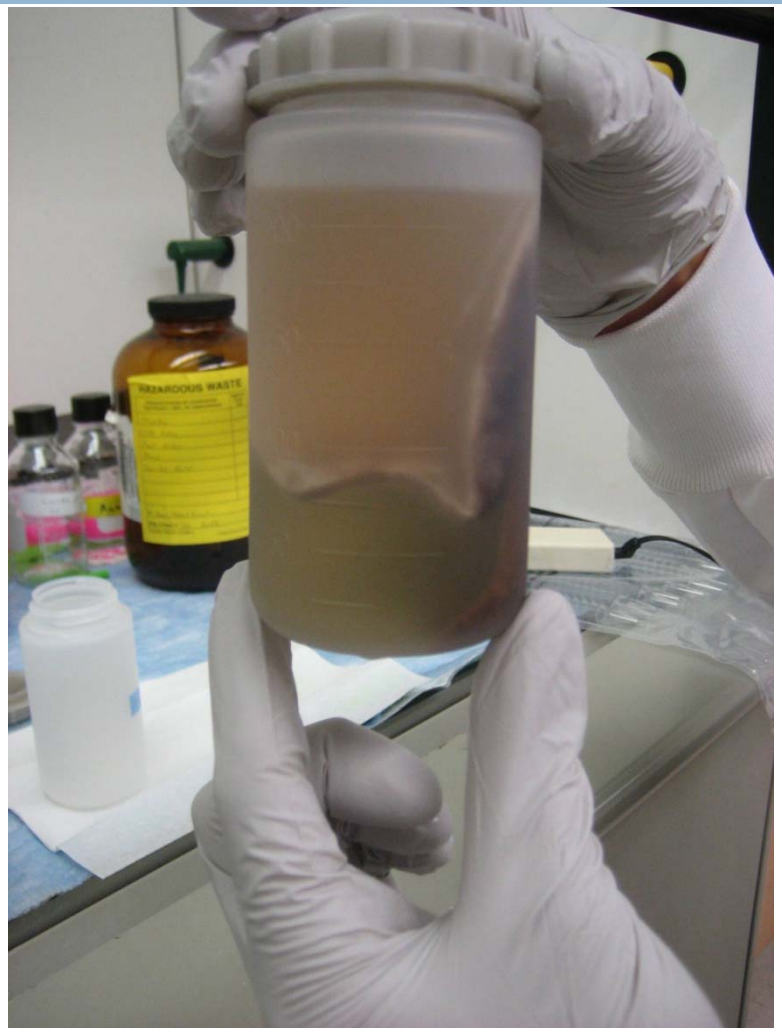
R^2: 0.988

Main point

- qPCR provides a potential method to obtain accurate, quantitative detection of *F. circinatum* from different substrates.

qPCR screening procedures

- Extract DNA from 400 slash pine seeds
  - 6 hours for 6 DNA extractions



qPCR method development

- Primers based on
  - Schweigkoffer, W., O'Donnell, K. and Garbelotto, M. 2004. Detection and Quantification of *Fusarium circinatum*, the Causal Agent of Pine Pitch Canker, from Two Calfornia Sites by Using Real-Time PCR Approach Combined with a Simple Spore trapping Method. Applied and Environmental Microbiology. Vol. 70 No. 6 3512-3520.
  - Produce a 360 bp product only in the presence of *F. circinatum* DNA
  - 5 hours to screen 6 seed lots

Time requirements

- qPCR can screen seeds in 2 days
- Culture-based method requires 2-3 weeks

qPCR machine

Current status

- Blotter Method
  - ID fungi from two seed lots
  - Inoculate seedlings with *F. circinatum* obtained from seeds
- qPCR method
  - Standardize DNA extractions
  - Run qPCR reactions on 6 seed lots
- All research should be completed by the end of summer

Potential applications

- Screen seeds before shipping internationally
- Screen seeds for potential damping off problems caused by *F. circinatum*
- Rapidly detect *F. circinatum* in diseased seedlings
- Compare cone/seed infections/contamination from different sources
- Use in epidemiology studies of *F. circinatum*

Thanks

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- Dr. Claire Anderson for technical assistance


Questions