Title: Citrus Greening is not Green!

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Abstract:

This action plan is designed to allow students to use newly obtained biotechnology lab skills within the context of a real-world problem. This is designed to be a project that encompasses prior skills and knowledge allowing students to discover problems for themselves and apply what they learn to a community awareness project.

The heart of this project will be Citrus Greening or Huanglongbing, a disease affecting citrus trees in the state of Florida. In this module students will study how the disease affects citrus trees; how to identify it morphologically and in the lab using an iodine test and PCR, study the cause and factors affecting its spread, track its spread within our state, and design a community awareness brochure. Tests on leaves and psyllids will be performed using kits available from Qiagen. Students will use statistics from government websites to determine how fast HLB is spreading and graphically display their results. Classes will take a field trip to a local citrus grove to observe, learn how to diagnose and identify affected trees, look for psyllids, and collect leaves for testing.

Rationale:

Students will have the opportunity to study Citrus Greening (Huanglongbing or HLB), a vector-borne bacterial disease that is devastating Florida's citrus industry at an alarming rate. Since it was first detected in Florida in 2005, HLB is now found in all of the states citrus producing counties. Once a tree has become infected, the fruit it produces becomes bitter and salty making it virtually inedible. Death for the tree is inevitable as there is no cure for this disease. The vector that carries and transmits HLB in Florida is a small Asian psyllid that can hop, fly, or be carried longer distances by winds. It is estimated that every citrus grove in the state is within one mile of an infected tree. According to the Florida Department of Citrus, the industry employs approximately 76,000 workers and has an annual economic impact of 9 billion dollars.[(1)] The United States leads the world in grapefruit production supplying the world with over 30% of its grapefruit, and is the third largest overall citrus producer in the world. The majority of the citrus grown in the United States comes with Florida as the major producer. In fact Florida produces three times as many tons of oranges and four times as many tons of grapefruit as its closest competitor, California.[(2)] With over ½ million acres of citrus groves and 74 million trees, Florida is second only to Brazil in orange juice production and supplies approximately 80% of the orange juice in the United States during any given growing season.[(3)] These facts clearly demonstrate that any threat to the Florida Citrus Industry is a threat to the world citrus supply.

This module would allow students to reinforce some laboratory skills and techniques they are already familiar with (electrophoresis, PCR) and use some that would be new to them (BLAST). In the past, lab skills have been used in an isolated fashion with various labs designed to teach the skill without a real-world frame of reference. This module would allow students to see how these seemingly isolated and unrelated skills work together to identify and diagnose HLB in citrus trees.

References Cited:

1. "Florida Grapefruit." Florida Department of Citrus. 2010. 30 Aug. 2011. <http://www.gofloridagrapefruit.com/about-fdoc/>.

2. "Background Statistics: U.S. Citrus Market ." United States Department of Agriculture. 22 Jan. 2007. 28 Aug. 2011. <http://www.ers.usda.gov/News/citruscoverage.htm>.

3. "Citrus Facts." Florida Citrus. 2008. 30 Aug. 2011. <http://www.floridajuice.com/juice.php>.

Lesson Plans: See Below

Day One: Students will do a webquest on Citrus Greening (Huanglongbing)

Day Two and Three: Discussion and Lecture on the citrus greening disease, it's vector the Asian Citrus Psyllid, and the Economic Impact for Florida using the following PowerPoint Presentations. These are excellent presentations with great graphics and include comments for the presenter.

1. ***The Asian Citrus Psyllid and the Citrus Disease Huanglongbing*** by the California Citrus Research Board http://casap.org/2009/2009conference/Asian_Citrus_Psyllid.ppt

2. ***The Asian Citrus Psyllid & Huanglongbing*** by Protect U.S. Community Invasive Species Network at the University of Florida http://entnemdept.ifas.ufl.edu/hodges/ProtectUs/Greening%205-12-2011.pdf

3. ***Three Exotic Plant Diseases Threatening Florida*** by the Florida State Agricultural Response Team http://www.flsart.org/ppt/Three%20Diseases.ppt

Day Four: Identify samples of citrus trees with HLB and compare to samples without HLB from a morphological perspective. If possible, taste test citrus that has been harvested from HLB infected trees. Discern whether or not they can tell infected Asian Citrus Psyllid from non-infected Psyllid by visual inspection under a stereoscope. Students will record their observations for each sample of leaf and psyllid and make their predictions as to infected vs. non-infected in a table.

Day Five: Students will use the "Iodine Starch Test" to further solidify or change their initial predictions from the previous day's inspections. This protocol is available from the University of Florida IFAS website http://edis.ifas.ufl.edu/hs375 .

Day Six: Students will begin preparation for PCR testing of their samples using the protocol provided by Dr. Kirsten Stelinski from UF.

Day Seven: Students will work on testing citrus samples and keep a record of results.

Day Eight: Continued as above

Day Nine: Students will graph results identified by location (city, county, etc.) and compare to actual data obtained from the State of Florida.

Day Ten: Students will develop informational flyers/brochures for Seminole County Residents (to be given out at Great Day in the County and local craft fairs) that include morphological detection methods and phone numbers to call for plant inspection and testing.

Day Fourteen: Field Trip to Lake Alfred

Materials and Supplies:

Thermocycler
Mortar and Pestle
1.5 mL microfuge tubes with caps
Autoclave
Stereoscopes
Hot Water Bath
Electrophoresis Apparatus
Microcentrifuge
Spatulas
Razor Blades

Chemicals Required:

Item	Source for Purchase	Catalog No.	Cost
Liquid Nitrogen	University of Central Florida Chemistry Department		Free
Absolute EMD Ethanol 500 mL	Fisher Scientific	NC9054029	$46.30
DNeasy Blood & Tissue Kit for 50 reactions	Qiagen		$144.00
DNeasy Plant MiniKit for 50 reactions	Qiagen		$193.00
Primer	IDT (Integrated DNA Technologies)		?
Loading Dye 1 mL 5x	BioRad	166-401 EDU	$50.00
Iodine Tincture or Tincture of Iodine or Lugol's solution	Drugstore Amazon		?
Buses for Field Trip			?
Total Cost			$433.30

Bibliography:

"Huanglongbing Florida Citrus Greening Information Video." World News, Inc.. 2011. 25 Aug. 2011. <http://wn.com/Huanglongbing_Florida_Citrus_Greening_Information_Video>.

Protocol: Purification of Total DNA from Plant Tissue
(Mini Protocol)
Provided by Dr. Kirsten Stelinski

Before starting:

Buffer AP1 may develop a yellow color upon storage. This does not affect the procedure.
All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.

Things to do before starting

- Buffer AP1 and Buffer AP3/E concentrate may form precipitates upon storage. If necessary, warm to 65°C to redissolve (before adding ethanol to Buffer AP3/E). Do not heat Buffer AP3/E after ethanol has been added.
- Buffer AW and Buffer AP3/E are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a water bath or heating block to 65°C.
- Label tubes with sample numbers/codes. You will need: 2 sets of 1.5 ul microcentrifuge tubes, 1 set of QIAshredder spin columns (lilac), and ones set of DNeasy Mini spin columns (white).
- Autoclave mortars, pestles, and spatulas for use in grinding plant tissue.

Procedure

1. Plant tissue (100 mg composite of petioles and midribs) can be ground to a fine powder under liquid nitrogen using a mortar and pestle. Allow the liquid nitrogen to evaporate and transfer the tissue powder to an appropriately sized tube. **Do not allow the sample to thaw.**

2. Add 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) to a maximum of 100 mg (wet weight) disrupted plant or fungal tissue and vortex vigorously. Spin briefly to remove any sample that has collected on lid of tube.
   No tissue clumps should be visible. Vortex or pipet further to remove any clumps. Clumps of tissue will not lyse properly and will therefore result in a lower yield of DNA.
   Note: Do not mix Buffer AP1 and RNase A before use.

3. Incubate the mixture for 10 min- 30 min at 65°C. Mix 2 or 3 times during incubation by inverting tube.
   This step lyses the cells.

4. Add 130 µl Buffer AP2 to the lysate, mix, and incubate for 5 min on ice.
   This step precipitates detergent, proteins, and polysaccharides.

5. Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm).

6. Pour the lysate (avoid transferring perllet) into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at 20,000 x g (14,000 rpm).
   It may be necessary to cut the end off the pipet tip to apply the lysate to the QIAshredder Mini spin column. The QIAshredder Mini spin column removes most precipitates and cell debris, but a small amount will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet in step 7.

7. Add 675 µl Buffer AP3/Eto a new tube (not supplied). Transfer the flow-through fraction from step 6 to the cleared lysate without disturbing the cell-debris pellet, and mix immediately by pipetting or gently vortexing.
Ensure that ethanol has been added to Buffer AP3/E.

8. Pipet 650 µl of the mixture from step 7, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube (supplied). Centrifuge for 1 min at _6000 x g (corresponds to _8000 rpm for most microcentrifuges), and discard the flow-through.* Reuse the collection tube in step 9.

9. Repeat step 14 with remaining sample. Discard flow-through* and collection tube.

10. Place the DNeasy Mini spin column into a new 2 ml collection tube (supplied), add 500 µl Buffer AW, and centrifuge for 1 min at 6000 x g (8000 rpm). Discard the flow-through and reuse the collection tube in step 11.
Note: Ensure that ethanol is added to Buffer AW.

11. Repeat step 10 by adding 500 µl Buffer AW to the DNeasy Mini spin column, and centrifuge for 2 min at 20,000 x g (14,000 rpm) to dry the membrane.
It is important to dry the membrane of the DNeasy Mini spin column since residual ethanol may interfere with subsequent reactions. Discard flow-through and collection tube.
Note: Following the centrifugation, remove the DNeasy Mini spin column from the collection tube carefully so the column does not come into contact with the flow through, as this will result in carryover of ethanol.

12. Transfer the DNeasy Mini spin column to a 1.5 ml or 2 ml microcentrifuge tube (not supplied), and pipet 50 µl Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature (15–25°C), and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute. Store at -20oC.

Conventional PCR to Examine the Presence of the HLB-associated Pathogen in Citrus:
Provided by Dr. Kirsten Stelinski

16S rDNA, OI1/OI2 specific to the '*Ca.* Liberibacter asiaticus' sp. used for PCR amplification in a 25-µl reaction volume

Forward primer: OI1 (5'-GCG CGT ATG CAA TAC GAG CGG CA-3')
Reverse primer: OI2c (5'-GCC TCG CGA CTT CGC AAC CCA T- 3')

PCR reaction
25-µl reaction volume consisting of:
1 µl of DNA template
200 nM (each) primer
200 µM (each) dNTPs
2.5 mM MgCl2
1× PCR buffer
dH_2O
1 unit Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA)

Amplification:
94°C for 2 min
35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min
Final extension cycle of 10 min at 72°C

Analysis of PCR reactions:

Expected product size: 1160 bp

1.0% agarose gel in 1× Tris-acetate-EDTA buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.5)

Any 1 kb DNA ladder should work e.g. Quick-Load® 1 kb DNA Ladder (New England Biolabs) or 1 kb Plus DNA ladder (Invitrogen)