Characterizing, detecting, and eliminating viruses to enable the safe introduction of seeds

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USDA-ARS, U. S. Vegetable Laboratory
Charleston, SC, USA

(International Seed Health Symposium-2015, Mexico)
As you can see here, there are ARS labs all over the country. There are divided into seven administrative areas based on the color shades. Dr. Gonsalves is in Hawaii, and my office is in Charleston, South Carolina. Here in Geneva, there is a USDA–ARS lab with two research units. For your students, there are job opportunities out there.
U. S. Vegetable Laboratory

The mission of the U.S. Vegetable laboratory is to improve genetic populations of vegetable crops on yield, quality and pest resistance; to generate knowledge on the etiology, ecology, epidemiology, & pathogenicity of plant pests, thus to develop new management strategies.
Major vegetable crops that we are working on: Brassica (Broccoli, Cabbage, Cauliflower, and Collared green), Solanaceous crops (Pepper and Tomato), Southern pea, Sweet potato and Watermelon. Today, I will focus my presentation on the emerging tomato diseases.
World Leading Tomato Producing Countries
(FAO statistics-2012, tonnes)
Top World Pepper Production –
FAO Statistics-2012 (tonnes)
Outline of the presentation

- Seed borne viral diseases on pepper, tomato and watermelon.
- Seed health testing technologies and their consideration.
- Strategies in seed-borne viral disease management.
Pepper Seed-borne Tobamoviruses

- **Family:** Virgaviridae
- **Genus:** Tobamovirus
- **Species:**
  - Pepper mild mottle virus (PMMoV)
  - Tobacco mosaic virus (TMV)
  - Tomato mosaic virus (ToMV)

(PMMoV + TMV)
Pepper mild mottle virus

PMMoV-pepper seed
Tomato Seed-borne Viruses and Viroids

- **Genus:** Tobamovirus
  - **Species:**
    - Tobacco mosaic virus (TMV)
    - Tomato mosaic virus (ToMV)
    - Tomato mottle mosaic virus (ToMMV)?

- **Genus:** Potexvirus
  - **Species:** Pepino mosaic virus

- **Genus:** Pospiviroid
  - **Species:** Potato spindle tuber viroid
Seed-borne viruses on cucurbits

SqMV: Squash mosaic virus
CGMMV: Cucumber green mottle mosaic virus
MNSV: Melon necrotic spot virus
Seedling growout for SqMV
CGMMV on greenhouse cucumber

(Photo courtesy Weizheng (John) Zhang)
Melon necrotic spot virus on cucumber

Marketer

Poinsett 76

Straight 8

MNSV, 5 DPI

Healthy
Outline of the presentation

- Seed borne viral diseases on pepper, tomato and watermelon.
- Seed health testing technologies and their consideration.
- Strategies in seed-borne viral disease management.
Virus structure, composition and the strategies in virus detection

Detection:
- Molecular (PCR)
- Serological (ELISA)

RNA or DNA

Coat protein

(From Agrios’ Plant Pathology, 3rd ed.)
Common Methods for Plant Virus Detection

**Indirect Tests (infectious + dead viruses)**

- Coat protein
- RNA or DNA

**Direct Tests (infectious)**

- Tobamovirus
  - (Local lesions)
- SqMV
  - (Growout)
Risk assessment:
Interpretation of seed health test results

- If following the standard method, a negative test result is used to certify a seed lot. Planting such certified seeds will likely not introduce that virus to a region or a country.

- Even if a seed-borne disease has established in a country, planting a certified virus-tested seed lot is still important to reduce initial virus inoculum, slow disease progression and minimize yield loss to an economic threshold level.
A right balance in seed health test is to prevent an infected seed lot to be traded in the market.

A false negative result may occur due to the poor sensitivity, specificity, or inhibition of a test.

A false positive result from the detection of dead or non-infectious viruses could result in an unnecessary rejection of a valuable seed lot.

Under this situation, particularly with a treated seed lot, the positive result from an indirect test (like ELISA, PCR) should be validated with a direct test to determine virus infectivity, i.e., seedling growout or bioassay, as appropriate.
Examples of seed health tests for seed-borne virus and viroid

- Pepino mosaic virus on tomato.

- Tobamoviruses (Pepper mild mottle virus) on pepper.

- Squash mosaic virus/Cucumber green mottle mosaic virus on cucurbits.

- Pospiviroids (Potato spindle tuber viroid) on tomato.
## Major seed-borne viruses and viroids on Solanaceous crops and seed health tests

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Host</th>
<th>Indirect test</th>
<th>Direct test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ELISA</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>Tobamovirus</td>
<td>PMMoV</td>
<td>Pepper</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TMV</td>
<td>Pepper/tomato</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>ToMV</td>
<td>Pepper/tomato</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Potexvirus</td>
<td>PepMV</td>
<td>Tomato</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pospiviroids</td>
<td>PSTVd/P CFVd</td>
<td>Pepper/tomato</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Bioassay with a local lesion test for tobamoviruses on tobacco plant

- ISTA/ISHI/NSHS: minimum 3,000 seeds in 250-500 seeds/subsamples (6-12 reps) through mechanical inoculation on tobacco, *Nicotiana tabacum* Xanthi NN.
- Induction of hypersensitive response reaction (necrotic lesions) on the inoculated resistant tobacco is a plant defense mechanism against virus attack.
Examples of seed health tests for seed-borne virus and viroid

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- Squash mosaic virus/Cucumber green mottle mosaic virus on cucurbits.
- Pospiviroids (Potato spindle tuber viroid) on tomato.
Pepino mosaic virus
Worldwide Distribution of PepMV
PepMV on seed coats, but not in embryos

Mechanical inoculation resulted in virus infection
Duo-Primers and a TaqMan Probe Design for Broad Spectrum Detection of PepMV

5’ACTCCTAGAGCTGACCTCAC

5’TGTCAGCTTTGATTACTTCCAAA

5’ACTCCTAGAGCTGACCTCAC

5’ATAGTTGGAACGACCTCT5’

CH-1
US-1
Fr
LE-00
LE-02
Sp13
SM. 74
CH-2
US-2

5’ACTCCTAGAGCTGACCTAC

5’t-g-t-a-c-a-c-c-ac

5’t-a-a-t-
Examples of seed health tests for seed-borne virus and viroid

- Pepino mosaic virus on tomato.
- Tobamoviruses (Pepper mild mottle virus) on pepper.
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- Pospiviroids (Potato spindle tuber viroid) on tomato.
Pepper mild mottle virus

- **Symptoms:** leaf chlorosis (*mild*), plant stunting, and distorted fruits (*severe*).

- **Spread:**
  - **Long distance:** Seed-borne (*outer seed coat*).
  - **Short distance:** mechanical transmission (hands, tools, clothing). Particular in greenhouse production.
  - **no insect vector. But by soil and water.** Humans as a vector (Stool and fecal pollution)?

- **Distribution:** Worldwide.
Management of PMMoV

- Host resistance ($L^1$, $L^2$, $L^3$, and $L^4$ R genes).
- Avoidance (clean seed), seed health test.
- Hygiene, sanitation and disinfection to limit secondary mechanical transmission.
- Rotation to a non-host crop, like tomato, eggplant.
Examples of seed health tests for seed-borne virus and viroid

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- Squash mosaic virus/Cucumber green mottle mosaic virus on cucurbits.
- Pospiviroids (Potato spindle tuber viroid) on tomato.
### Cucurbit seed-borne viruses

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Seed health test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Indirect Test</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ELISA</td>
</tr>
<tr>
<td>Comovirus</td>
<td>SqMV</td>
<td>+</td>
</tr>
<tr>
<td>Carmovirus</td>
<td>MNSV</td>
<td>+</td>
</tr>
<tr>
<td>Tobamovirus</td>
<td>CGMMV</td>
<td>+</td>
</tr>
</tbody>
</table>

- ISTA/ISHI: ELISA with minimum 2,000 seeds in 20 subsamples.
- With the recent outbreaks of CGMMV in Australia, the requirement for seed health test in Australia has been increased to 9,400 seeds.
Genetic diversity of CGMMV isolates in the world
RT-LAMP Product Detection for CGMMV

Sensitivity

Specificity

SYBR Green I
<table>
<thead>
<tr>
<th>Sample</th>
<th>Cycle Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH sample 1</td>
<td>16.04</td>
</tr>
<tr>
<td>GH sample 2</td>
<td>16.14</td>
</tr>
<tr>
<td>V13-164</td>
<td>8.98</td>
</tr>
<tr>
<td>V13-165</td>
<td>18.91</td>
</tr>
<tr>
<td>V13-166</td>
<td>11.44</td>
</tr>
<tr>
<td>V13-167</td>
<td>21.2</td>
</tr>
<tr>
<td>V13-168</td>
<td>25.73</td>
</tr>
<tr>
<td>V13-169</td>
<td>22.24</td>
</tr>
<tr>
<td>V13-170</td>
<td>11.62</td>
</tr>
<tr>
<td>V13-171</td>
<td>12.05</td>
</tr>
<tr>
<td>WATER</td>
<td>36.35</td>
</tr>
</tbody>
</table>

**CGMMV**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cycle Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH sample 1</td>
<td>No Ct</td>
</tr>
<tr>
<td>GH sample 2</td>
<td>No Ct</td>
</tr>
<tr>
<td>V13-164</td>
<td>30.17</td>
</tr>
<tr>
<td>V13-165</td>
<td>14.52</td>
</tr>
<tr>
<td>V13-166</td>
<td>26.83</td>
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<tr>
<td>V13-167</td>
<td>35.54</td>
</tr>
<tr>
<td>V13-168</td>
<td>20.68</td>
</tr>
<tr>
<td>V13-169</td>
<td>9.19</td>
</tr>
<tr>
<td>V13-170</td>
<td>35.41</td>
</tr>
<tr>
<td>V13-171</td>
<td>17.66</td>
</tr>
<tr>
<td>WATER</td>
<td>No Ct</td>
</tr>
</tbody>
</table>

**MNSV**
Seed health assay – SqMV

- Molecular characterization and understanding the genetic diversity.
- Serological specificity ofSqMV strains.
- Improvement to primer and probe design to achieve a broad spectrum detection of SqMV using real-time RT-PCR for reliable seed health test.
SqMV Genetic Diversity

Nucleotide Substitution per 100 residues

Genotype I
- M96148_H-SqMV_CP
- I20364_H-SqMV_CP
- AF059533_K-SqMV_RNA2
- AB054689_Y-SqMV_RNA2
- EU421060_CH99-211
- DQ868881_CH99_211cp
- AF059532__Z-SqMV_RNA2

Genotype II

Genotype III

Nucleotide Substitution per 100 residues

12.3
12 10 8 6 4 2 0
Improvement to qRT-PCR in SqMV

SqMV-2011 qRT-PCR

Type I (CA-SqMV)

CTRL

Type III (RZ-SqMV)

CTRL

(No reaction to Type III)

SqMV-2015 qRT-PCR

Type I (CA-SqMV)

CTRL

Type III (RZ-SqMV)

CTRL

(Broad reaction to Types I and III)
Seed health testing using duplex qRT-PCR with an internal control to detect SqMV on melon

<table>
<thead>
<tr>
<th></th>
<th>Means Ct</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>18S rRNA</td>
</tr>
<tr>
<td>5 seeds</td>
<td>+</td>
</tr>
<tr>
<td>10 seeds</td>
<td>+</td>
</tr>
<tr>
<td>100 seeds</td>
<td>+</td>
</tr>
<tr>
<td>Healthy CK-</td>
<td>+</td>
</tr>
<tr>
<td>Positive CK+</td>
<td>+</td>
</tr>
</tbody>
</table>

The internal control (18S rRNA) is to determine the quality of sample preparation and amplification without inhibition to enzyme activities.
Comparison of new qRT-PCR (SqMV_2015) and the old qRT-PCR (SqMV_2011) for detection of SqMV

<table>
<thead>
<tr>
<th>Crop and tissue</th>
<th>Country of origin</th>
<th>New qRT-PCR (SqMV_2015)</th>
<th>Old qRT-PCR (SqMV_2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melon seed</td>
<td>Peru</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melon seed</td>
<td>Peru</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melon seed</td>
<td>Peru</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melon seed</td>
<td>Peru</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melon seed</td>
<td>Peru</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melon seed</td>
<td>Peru</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rootstock seed</td>
<td>Netherlands</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rootstock leaf</td>
<td>Netherlands</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melon leaf</td>
<td>Spain</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Melon leaf</td>
<td>Spain</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melon seed</td>
<td>Peru</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Squash leaf</td>
<td>Spain</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Non-Template</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
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Greenhouse Tomato Viroid Disease

Tomato chlorotic dwarf viroid
Mexican papita viroid infection
Natural Tomato Infecting Viroids in North America

- **Potato spindle tuber viroid**
  - California, North Carolina

- **Tomato chlorotic dwarf viroid**
  - Canada, Arizona, Mexico

- **Mexican papita viroid**
  - Canada, Mexico

- **Citrus exocortis viroid?**

Nucleotide Substitution per 100 residues

22.6

20 15 10 5 0
Transmission of PSTVd in Tomato Seeds in seedling grow-out and mechanical transmission

For seed health test, Australia is requiring testing of 20,000 seeds/lot in 400 seeds/subsample, that is 50 subsamples/lot.
Developing Seed Health Tests

PSTVd in 1, 5, 10 seeds

PSTVd in 10 single seed

PSTVd in 1 seed mixed with clean ones (0, 100, 200, 400, 800)

PSTVd in 1 seed mixed with clean ones (5 replicates of 400 seeds)
Outline of the presentation

- Seed borne viral diseases on pepper, tomato and watermelon.
- Seed health testing technologies and their consideration.
- Strategies in seed-borne viral disease management.
Nursery plants

Bumble bees

Intercropping

De-leafing
Integrated Disease Management to manage seed-borne viruses and viroids on vegetable crops

- Quarantine (CGMMV).
- Selecting disease resistant cultivars.
- Planting certified virus-tested clean seeds.
- Disinfectants to limit mechanical transmission of seed-borne viruses (PepMV, TMV, ToMV and PSTVd).

S        R         S         R
Selected Targets of Greenhouse Tomato Viruses/Viroid

- Pepino mosaic virus (PepMV)
- Tomato mosaic virus (ToMV)
- Tobacco mosaic virus (TMV)
- Potato spindle tube viroid (PSTVd)
Most Effective Disinfectants for ToMV/TMV

10% Bleach  
20% NFDM  
50% Lysol
Efficacy of Virkon-S Concentration on TMV Infection
Efficacy of Disinfectant Solutions Upon Storage

A

10% Clorox

TMV only
Fresh
30 days

B

2% Virkon-S

TMV only
Fresh
10 days
30 days
### Most effective disinfectants against major greenhouse tomato Viruses/Viroid

<table>
<thead>
<tr>
<th>Disinfectants</th>
<th>PepMV</th>
<th>ToMV</th>
<th>TMV</th>
<th>PSTVd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clorox (10%)</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Virkon S (2%)</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>NFD Milk (20%)</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Lysol (50%)</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>Partial</td>
</tr>
</tbody>
</table>
Summary

- Planting a certified virus-tested seed lot is an important preventative measure against seed-borne viruses.

- A reliable seed health test should be sensitive in detecting the presence of virus particles, but also need to determine the virus infectivity through appropriate bioassay, particularly on those treated seed lots.

- An integrated disease management should be employed, from planting a virus-resistant cultivar, to the use of effective disinfectants, and crop rotation to non-host plants.
Acknowledgments

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Weizheng (John) Zhang, Alberta Agriculture and Forestry (photos on CGMMV).