Phytoplasma Diseases in Citrus Orchards of Pakistan

Shazia Mannan
COMSATS Institute of Information Technology
Sahiwal Campus, Pakistan
Citrus in Pakistan

• Citrus is one of the major export commodities of Pakistan and is grown in an area of 160,000 ha.

• Annual production of citrus is 1.5 MMT (http://www.pakissan.com/english/allabout/orchards/citrus/index.shtml).

• Most of this citrus is grown in the province of Punjab including the Districts of Sargodha, Sahiwal, Lahore, Sialkot, Jhang, Mianwali, Multan and Gujranwala.
Citrus Varieties

Following are the main commercial varieties of citrus in Pakistan:

- **Mandarines**: Feutrells Early and Kinnow
- **Grape Fruit**: Mash Seedless, Duncan, Foster and Shamber
- **Lemon**: Eureka, Lisbon Lemon and rough Lemon
- **Lime**: Kaghzi Lime and Sweet Lime
Phytoplasma Diseases of Citrus

- Witch’s Broom Disease of Lime
- Little Leaf Disease of Citrus
- Stubborn Disease
In 1970, Chapot surveyed Pakistan and he reported wide occurrence of citrus stubborn disease symptoms (Chapot, 1970).

In 2009 Phytoplasma was detected in orchards near Islamabad (Fauqia, 2009).

Phytoplasmas have been reported to cause losses of about 70-100% in the majority of earning crops (Armando et al., 2005).
Objectives of Present Study

- Detection of phytoplasmas in citrus orchards of southern Punjab province of Pakistan
- Identification of possible alternative hosts
- Identification of insect vector(s)
Surveys of citrus Areas
Sampling Strategy

- In the districts of Sahiwal, Pakpattan and Multan all the tehsils were visited.
- In each tehsils, 5 orchards were visited.
- From each orchard at least 5 symptomatic samples were collected.
- Numbers of orchards for sampling depended upon cropping intensity.
- More orchards were visited in tehsils with intense cropping such as in Sahiwal district.
- Orchards of at least one acre area were observed for phytoplasma symptoms and sample collection.
Sample Collection

Citrus Samples

Leaves showing phytoplasma-like symptoms were collected from plants including:

- Sweet Oranges
- Blood oranges
- Grapefruit
- Mandarins
- Lemons
- Limes
Weed Sampling

In order to identify possible alternative hosts following weeds were collected:

- Couch grass *Cynodon dactylon* (L.) Pers.
- Wild oat *Avena fatua* L.
- Field bindweed *Convolvulus arvensis* L.
- Fat-hen *Chenopodium album*.
Insect Sampling

Following Insects were collected from around the plants showing phytoplasma-like disease symptoms:

• **Asiatic citrus psylla**
  *Diaphorina citri* Kuwayama
  [Hemiptera: Psyllidae]

• **Leafhoppers**
  *Balclutha punctata* (Fabricius)
  *Empoasca decipiens* Paoli
  [Hemiptera: Cicadellidae]
DNA Extraction

DNA was extracted and purified from 1 g of petioles and midribs using the CTAB method described by Doyle and Doyle (1990)
Sample Analysis

Total 20 samples of sweet orange were tested from Sahiwal district:

- 15 from farmers’ orchards
- 5 from the Horticulture Research Center at Sahiwal.
Phytoplasma DNA PCR

• **Single PCR** with O-MLO primers of Doyle and Doyle (1990)

• **Nested PCR** The P1/P7 primer pair of Deng and Hiruki (1991) and Schneider et al., (1995) were used in conjunction with primers R16F2n/ R16R2 and R16mF2/ R16mR1 (Gundersen and Lee, 1996).

* DNA of ‘*Candidatus* Phytoplasma urantifolia’, obtained from Central Science Laboratory, UK, was used as a positive control during amplifications.
## Sequences of Primers

<table>
<thead>
<tr>
<th>Title</th>
<th>Sequence (5´ to 3´)</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
<td>AAGAGTTTGATCCTGGCTCAGGATT</td>
</tr>
<tr>
<td>P7</td>
<td>CGTCCTTCATCGGCTCTTT</td>
</tr>
<tr>
<td>R16F2n</td>
<td>GAAACGACTGCTAAGACTGG</td>
</tr>
<tr>
<td>R16R2</td>
<td>TGACGGGGCGGTGTTGTGTAACAAACCCCG</td>
</tr>
<tr>
<td>O-MLO-F</td>
<td>ACGAAAGCGTGGGGAGCAAA</td>
</tr>
<tr>
<td>O-MLO-R</td>
<td>GAAGTCGAGTTGCAGACGCC</td>
</tr>
<tr>
<td>R16mF2</td>
<td>CATGCAAGTCGAACGA</td>
</tr>
<tr>
<td>R16mR1</td>
<td>CTAAACCCCAATCATCGAC</td>
</tr>
</tbody>
</table>
Position of Primers on Phytoplasma rRNA Operon

16S region

Spacer Region

23S region

5’

1.2kb

1.8kb

R16mF2

R16mR1

P1

P7

1.2kb
Results

6 samples from farmers’ orchards and 3 from the Center were found to be infected.

**Single PCR** amplified a 558 bp sequence from the phytoplasma 16S rRNA gene of DNA extracted from infected plants.

**Nested PCR** amplified a 1.2 kb fragment confirming infection with a phytoplasma (Fig. 1).

The amplicons will be sequenced to determine which group the phytoplasmas belong to.

* Screening of the insects as well as the weeds collected from the orchards is in progress!
PCR Results

Lanes 1-3: Single PCR
Lanes 4-7: Nested PCR
Lanes 1, 2 & 4-6: Infected sweet orange
Lanes 3 & 7: Control (‘Ca. Phytoplasma aurantifolia’)
Lane 8: Molecular weight markers
CONCLUSION

- Infection with Phytoplasmas appears to be common in the south of Punjab province of Pakistan
Future Plans

- Detection and characterization of citrus phytoplasma from all provinces of Pakistan
- Identification of strains of phytoplasma prevalent in different varieties of citrus in Pakistan
Recommendations

This project will provide information that can be used to help control phytoplasma diseases by:

★ Implementation of these studies in other citrus growing areas.

★ Ensuring Healthy bud wood supply through screening of mother plots at Citrus Resource Centers throughout the country.

★ Keeping Orchards free from weeds that serve as alternate hosts for phytoplasma.

★ Applying special control strategies against the insects identified as vectors for phytoplasma.
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Thank You