Bacillus thuringiensis translocation inside citrus plants and insecticidal activity against Diaphorina citri, vector of HLB causal agents

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• Citrus is one of the most economically important fruit crops in the world.

Of every 5 cups of orange juice consumed in the world, 3 are Brazilians. (Neves et al., 2010)
• Citrus are perennial plants and they suffer in the field…

**High number of pathogens and pests**

*Cause losses in citrus production*
• HLB affects all citrus commercial varieties.

• HLB causal agent is a non-cultured, sieve phloem-restricted member of the a subdivision of the Proteobacteria: There are three species of ‘Candidatus Liberibacter’. ‘Ca. Liberibacter africanus’ in Africa, ‘Ca. Liberibacter asiaticus’ in Asia and America and ‘Ca. Liberibacter americanus’, in Brazil (less frequent in the orchards).
Some HLB symptoms are yellow shoots, leaf blotchy mottle, lopsided fruit with color inversion, and aborted seeds, however they do not always occur on the same tree at once; they can be distorted or masked by symptoms of other diseases, or induced by causes other than HLB.
**Diaphorina citri** (Hemiptera: Liviidae)

**Asian citrus psyllid (ACP) live cycle**

- **Eggs**: 2.6-8.2 days
  - 0.3mm
  - 200-800
- **Nymphs**: 10.7-39.3 days
- **Adult**: 2-3mm
- **Live cycle**: 15-45 days

*Picture: Cunha, T.*
• Conventional control → chemical insecticides

• During the last years, research has been focusing on developing less aggressive ACP and HLB management programs as:
  
  • Biological control by natural enemies (e.g. *T. radiata*) and entomopathogens (e.g. *I. fumosorosea*).

  • Biotechnology strategies (e.g. RNAi and GMO).
Bacillus thuringiensis (Bt)

- Widely used in agriculture;
- Gram positive;
- Rod-shaped bacteria;
- Aerobic (facultative anaerobiosis);
- Found for the first time in 1901 in Japan on silkworm larvae (Bombyx mori);
- Berliner in 1911 - Anagasta kuehniella;
- Synthesis of δ-endotoxins or protoxins (Cry Toxin)

(Praça et al., 2007; Bideshi et al., 2013)
**Bt action mechanism (Cry toxin)**

*Bacillus thuringiensis (Bt)*

- **Solubilization**
- **Activation**

**Ingestion**

- **Cell death**
  - **Septicemia**
  - **Dead larvae**

**Insect midgut cells**

- **Binding to receptor**
- **Toxin monomer**
- **Toxin oligomer**
- **GPI-anchored protein**
- **Membrane insertion**
- **Pores lead to osmotic cell lysis**

**Cadherin**

- **Activation of cell death pathway**

Jurat-Fuentes Laboratory
(http://web.utk.edu/~jurat/)
How we can control *Diaphorina citri*, which is a phloem-sucking insect?

Chewing insect pests

*Helicoverpa* sp.
Introduction

Confocal microscopy images of cabbage leaves. Leaves were taken from cabbage plants 48 h after root inoculation with sterile NYSM medium (controls: A–C) or Btk::GFP (D–I). Images show: autofluorescence of plant tissue alone at 515 nm (A, D, G); fluorescence at 594 nm due to anti-GFP antibody (B, E, H); merged images of autofluorescence and antibody fluorescence (C, F, I). The following structures are labelled: c, chloroplasts; p, parenchyma; v, vein.

Monnerat et al., (2003; 2009)

Localization of Btk::GFP in petiole. The arrow indicates the presence of Btk::GFP visible in the Xylem of the plant. Fluorescence signal as well as its movement in the xylem was used to ascertain the presence of the bacterium. Many Btk::GFP were visible in the samples although only a few were seen at any time at high resolution due to the narrow focal plane.

A and B. Plants of cotton and cabbage respectively, inoculated with supernatant of the final wash of Bt culture.
Balbinotte (2011) and Dorta (2014)

• 3 Bt strains (wild type with more than one cry or cyt toxin) + 10 recombinant strains

*Murraya paniculata* in suspension of Bt - Bioassay developed for nymphs of *D. citri* (A); 3rd instar nymphs after 24 h of transfer (B); Nymphs of *D. citri* in transition from the 3rd to the 4th instar, and exhibiting the descent behavior for the branch stem, natural at this stage (C) (BALBINOTTE, 2011)

Evaluation of *D. citri* nymphs survival after 120 hours of inoculation with Bt strains. A) Dead nymph, B) Live and dead nymphs, C) Live nymphs (DORTA, 2014)
1. Demonstrate Bt translocation inside sweet orange seedlings;

2. Confirm the mortality caused by the Bt strains previously identified as pathogenic to *D. citri*;

3. Select the best Bt strains to control ACP.
Materials & Methods

1. **Bt cultivation and inoculation**

- **Filter paper strip with Bt spores**
- **Bt strains suspension (NYSM medium)**
- **Bt strains spores and crystal confirmation**
- **Adjustment of Bt concentrations with the Neubauer chamber**
- **Bt root inoculation (substrate inoculation)**
  - Different concentrations (10 mL)

28 °C to 200 rpm for 72 hours
Materials & Methods

2. Bt translocation and detection

Bt isolation in NYSM medium

Leaves (shoots)
Dead nymphs

Bt strains inoculation (isolation after 5 days)

Bt colonies PCR confirmation

Specific primers

Fluorescence microscopy confirmation

Btk:GFP inoculation
(Samples were analyzed under fluorescence microscopy after 24 h)

Treated and untreated citrus seedlings
Results

2. Btt translocation (Stem)

Figure 5. Btk-GFP translocation in Sweet Orange seedlings. Samples of stem (1) and root (2) analyzed after 24 hours of inoculation with the bacterium or control treatment (water) in a 1000-fold increase. (A) Microscopy without fluorescence, (B) autofluorescence of the plant (wavelength 530-550 nm), (C) red fluorescence (wavelength 594 nm), (D) Composite image and (E) Uninoculated control.
Figure 5. Btk-GFP translocation in Sweet Orange seedlings. Samples of stem (1) and root (2) analyzed after 24 hours of inoculation with the bacterium or control treatment (water) in a 1000-fold increase. (A) Microscopy without fluorescence, (B) autofluorescence of the plant (wavelength 530-550 nm), (C) red fluorescence (wavelength 594 nm), (D) Composite image and (E) Uninoculated control.
Results

2. Bt detection

Figure 4. Agarose gel (1%) with PCR products from Bt colonies. (A) Detection of genes presented in S1 and S2 strains. (B) Detection of the gene presented in the Tox9 (recombinant) and S3 strains. L-ladder 1 Kb. N (pl) - Plant DNA negative control. N (Bt) - Bt DNA negative control. The numbers 1 to 16 correspond to Bt colonies from leaves or death nymphs.

100% of citrus seedlings and dead nymphs were confirmed with Bt strains presence.
3. Pathogenic assays

1. 6 seedlings of *Citrus sinensis* were used per treatment.

2. 10 *D. citri* nymphs (3\textsuperscript{rd} stage) were transferred to each seedling.

3. Treatments application (10 mL from each suspensions of Bt strains, and distilled water as control)

4. The nymph’s mortality evaluation (every 24 hours up to 120 hours after treatments application).
3. Assays with *D. citri* nymphs on citrus seedlings

- Preliminary screening with 8 Bt strains – 4 strains (S1, S2, Rec5 and Tox9) provided nymph’s mortality around 70%.

![Screening with 4 best Bt strains](image)

**Figure 1.** Visual evaluation of the mortality of *D. citri* nymphs in a stereoscopic microscope (Tecnival), after 120 hours of the treatments. (A) Control - not inoculated, (B) Bt treatment.

*Mean values marked with the same letter are not significantly different according to Tukey test (P < 0.05).*
3. Assays with *D. citri* nymphs on citrus seedlings

**Results**

**2 Bt strains**
**3 concentrations**

**Number of live nymphs after 120 h**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of live nymphs after 120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
</tr>
<tr>
<td>S1 (10^{10})</td>
<td>2</td>
</tr>
<tr>
<td>S1 (10^{9})</td>
<td>2</td>
</tr>
<tr>
<td>S1 (10^{8})</td>
<td>2</td>
</tr>
<tr>
<td>S2 (10^{10})</td>
<td>1</td>
</tr>
<tr>
<td>S2 (10^{9})</td>
<td>1</td>
</tr>
<tr>
<td>S2 (10^{8})</td>
<td>1</td>
</tr>
</tbody>
</table>

Mean values marked with the same letter are not significantly different according to Tukey test (P < 0.05).

**Figure 2.** Visual evaluation of the mortality of *D. citri* nymphs in a stereoscopic microscope (Tecnival), after 120 hours of the treatments. (A) Control - not inoculated, (B) Bt treatment.

**Experimental design**
6 replicates per treatment
Assay repeated twice
• The Btk:GFP was confirmed in all sweet orange seedlings used in the translocation assays.

• The presence of the bacteria was confirmed in young leaves and dead nymphs through isolation in NYMS medium and PCR detection with specific primers.

• Eight Bt strains were evaluated and three of those showed nymphs mortality above 60%.

• S1 and S2 strains were the best treatments in controlling ACP.
• Test the S1 and S2 strains with citrus nursery trees;

• Understand how the citrus trees take up the Bt strains;

• Evaluate the Bt strains under different citrus scion-rootstock combinations;

• Study the Cry toxins action mechanisms in the *D. citri* midgut.
Thank you!

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