HLB infection in *Murraya* species and related studies

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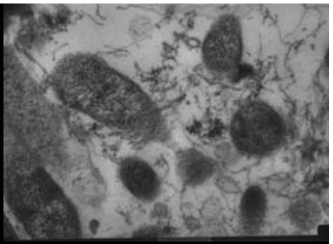
Why Study Alternative Hosts of HLB?

- Important to any eradication or control effort to understand if various rutaceous plant species are susceptible to Las, Lam, and Laf
- It is equally important to know if susceptible rutaceous hosts can serve as reservoirs of HLB, by acting as a source for transmission back to citrus
- It's also important to know what role these species play in the life cycle of the psyllid

Alternative Hosts of HLB: Objectives



- Determine the susceptibility of various rutaceous plant species to Las, Lam, and Laf and the psyllid transmission from these hosts to citrus
- Determine if rutaceous hosts can serve as reservoirs of HLB
- Determine if passage through alternative hosts affects the biology (pathogenicity) of the pathogen

Identification of Alternative Hosts

17 plant species listed as psyllid hosts and/or symptomatic hosts for the HLB bacterium will be tested:

Atalantia, ***Citrus jambhiri,*** *C. obovodoidea, C. limetta, C. macroptera, C. indica, Clausena anisum-olens, Clausena indica, Clausena lansium, Eremocitrus glauca,* ***Murraya exotica, Murraya paniculata, Murraya (Bergera) koenigii, Severinia buxifloia,*** *Toddalia asiatica, Vepris lanceolata and Zanthoxylum fagara,* ***Z. clava-herculis*** and *Z. hirsutum*

Vectors, hosts and HLB isolates

Diaphorina citri (Asian citrus psyllid) colonies were obtained and maintained on healthy sweet oranges.

Two isolates of Ca. Liberibacter asiaticus were started in sweet orange, one from Taiwan and one from Florida.

Rutaceous host plants were obtained from commercial suppliers and pre-tested for the presence of HLB

Materials and Methods

Diaphorina citri were tested for HLB using real-time PCR.

Sweet orange source plants were tested for HLB using the same assay.

HLB negative psyllids were transferred to infected oranges for 21 days.

After this acquisition period 10 individual psyllids were assayed by real-time PCR to assure acquisition of the bacteria was successful.

Materials and Methods

HLB positive *Diaphorina citri* were transferred to healthy test plants for 14 days.

Following the inoculation period psyllids were removed from the test plants, and 10 individual psyllids were tested for the presence of HLB.

The test plants were assayed for the presence of HLB at multiple time points following inoculation, continuing until all plants tested negative or 32 months, whichever came first.

Back assay Materials and Methods

Alternative hosts that tested positive for HLB were tested as potential sources by back-inoculation to orange

Psyllids were placed on infected plants for 14 days, tested for HLB by real-time PCR, and transferred to healthy sweet orange for 14 days

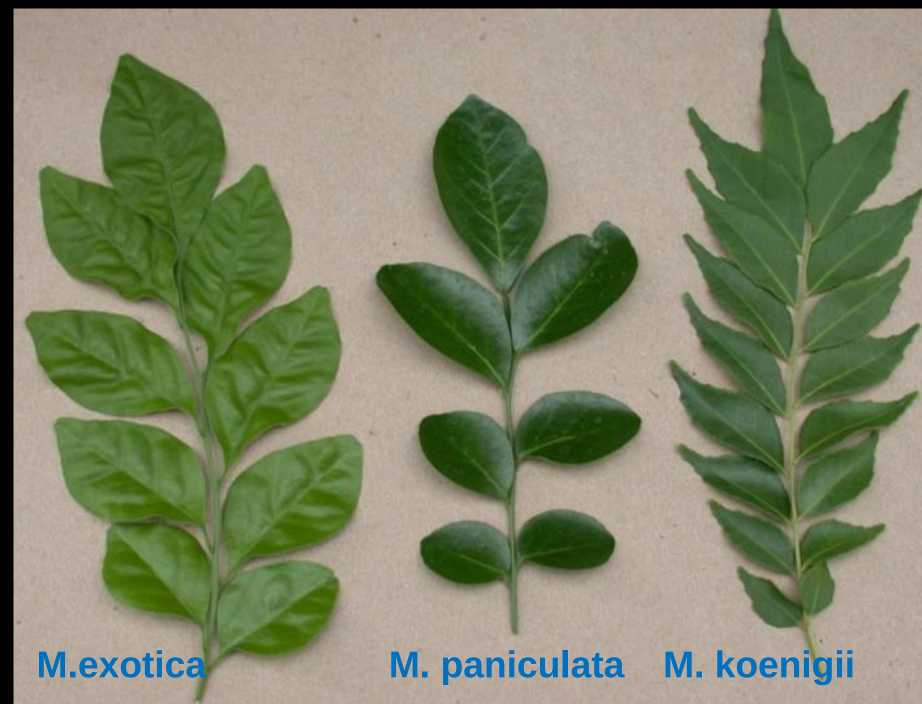
Inoculated sweet oranges were tested for HLB at multiple time points following inoculation

Murraya species

- Commonly used ornamentals and spice source grown in Florida
- 3 species: *M. paniculata*, *M. exotica*, and *Bergera (Murraya) koenigii*
- M. paniculata and M. exotica previously described as hosts

Murraya species

M. exotica M. koenigii



Murraya paniculata **inoculation with** Las

- Psyllids on HLB sweet orange (avg Ct 24.6) Allowed to feed and multiply for 3 wks
- 10 individual psyllids tested by qPCR with Ct values ranging from 14.4 to 22.1
- Psyllids were used to inoculate the *Murraya spp.*
- 14-day IAP on *M. paniculata*, 6-10 surviving psyllids were tested; Ct values ranged from 14.4 to 39.1 (3 =0)

Murraya paniculata

34 out of 36 inoculated plants were postive with Ct values ranging from 29.1 to 36.4

18 of the positive plants had values below 32

Both Florida and Taiwan isolates of HLB were able to infect *M. paniculata*

Presence of HLB confirmed by sequencing of ITS region from infected plants

HLB Infected *Murraya paniculata* showed no apparent symptoms

HLB persistence in *Murraya paniculata*

After 32 months only 12 remaining plants were positive by real time PCR, and of these none were below the Ct threshold of 32

The infection could be boosted by a re-inoculation 30 months after the original inoculaiton

There was an apparent lack of fitness of the bacterium in the plants

Murraya paniculata as a source of HLB

After a couple months eight infected Murraya paniculata were chosen as source plants for a back inoculation to sweet orange. The Ct values for these plants ranges from 29.1 to 34.6

HLB Transmission: *M. paniculata* to Citrus

M. paniculata Plant #/Ct	Number *D. citri* on/off	*D. citri* Post-AAP (Ct)	Sweet orange 3 month PI (Ct)	Sweet orange 22 month PI (Ct)	Sweet orange symptoms
2[a]/(33.4)[b]	40/27[c]	23.2[d]	23.1[e]	21.1	+
3/(34.8)	40/17	32.4	23.2	22.1	+
4/(32.8)	40/24	39.1	0.0	0.0	-
10/(34.5)	40/34	39.2	38.4	0.0	-
11/(34.9)	40/38	34.8	38.5	38.7	-
*13/(29.1)	40/38	31.4	36.8	41.0	-
*15/(32.7)	40/31	--[f]	38.8	0.0	-
*17/(32.6)	40/36	--	32.3	--	-
*18/(32.9)	40/33	--	38.8	--	-
*19/(31.2)	40/30	--	39.9	0.0	-

Murraya exotica

- 23 plants inoculated, 21 tested positive for HLB one month post-inoculation with Ct values ranging from 19.6-36.9
- 3 months post inoculation the Ct values had dropped, ranging from 33.6-38.9
- By 7 months post inoculation all plants were negative
- The presence of HLB was confirmed by sequencing of the ITS region

Infected *Murraya exotica* show no symptoms

Bergera (Murraya) koenigii

- 17 plants inoculated
- 4 of the 17 plants tested positive by real-time PCR, with Ct values ranging from 32.2 to 40.2
- No PCR products typical of Las could be sequenced, despite multiple efforts using multiple primer sets
- These plants were eventually determined to be negative for HLB

Zanthoxylum beechyanum

Zanthoxylum

- 3 plants inoculated by psyllids
- None of the plants tested positive by real-time PCR

Psyllids would live and feed on
- Zanthoxylum, but would not reproduce.

7 Zanthoxylum were graft inoculatd with infected sweet orange, these were also negative

Conclusions

- *M. paniculata* is an excellent host for the psyllid; quite possible as a reservoir host of the HLB-associated bacterium.
- There is successful transmission from *M. paniculata* back to citrus.
- *M. paniculata* and *M. exotica* could serve as bridging hosts of Las
- *Bergera koenigii* is not a host
- Zanthozylum is not a host

Additional projects: Improved DNA extraction from psyllids

Traditional psyllid extraction methods required two days for 29 extractions

The new high throughput method required 2 hours for a 96 samples, and resulted in greater sensitivity

A new DNA extraction technique was developed for psyllids, utilizing steel beads in a 96 well plate format

Additional projects: HLB population complexity

- Cloning populations instead of direct sequencing led to the discovery of complex HLB populations

- Mixed infections of Las and Lam were common, with Las tending to dominate the infection

- Mixed infections of Las and Laf also occurred, but no Las dominance was observed

- Occasionally individual plants were infected with all three Liberibacters

HLB population complexity: Las + Laf mixed infection

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                        310       320       330       340       350       360       370       380
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Las_DQ778016      TGTTGAGTATCATTGAATTTATTGAGTGATCTGAACGTTTTTGAAGATTAAAGCTTTTAATTAAGCTTGATATAAATT
B436_2            ...............................................................................
BZ913_37          ...............................................................................
BZ918_1           ...............................................................................
BZ921_46          ...............................................................................
US1_21            ...............................................................................
India_39          ...............................................................................
Indo_5            .............................................G.................................
PRC2              ...............................................................................
Viet_19           ...............................................................................
Laf_EU754741      .A........T...AG.......T.A.........TA......A.......---------------T......C..TA.
AF227_10          .A........T...AG.......T.A.........TA......A.......---------------T......C..TA.
AF229_6           .A........T...AG.......T.A.........TA......A.......---------------T......C..TA.
BZ913_32          .A........T...AG.......T.A.........TA......A.......---------------T......C..TA.
BZ918_40          .A........T...AG.......T.A.........TA......A.......---------------T......C..TA.
B436_6            .A........T...AG.......T.A.........TA......A.......---------------T......C..TA.
US1_8             .A........T...AG.......T.A.........TA......A.......---------------T......C..TA.
India_15          .A........T...AG.......T.A.........TA......A.......---------------T......C..TA.
Lam_AY859542      ....T.T...T...AG...A...TGA....A.T.G.A......A.....AG.GCG..AAGC.----.AA.....G.TC.
BZ941_43          ....T.T...T...AG...A...TGA....A.T.G.A......A.G...AG.GCG..AAGC.----.AA.....G.TC.
BZ941_45          ....T.T...T...AG...A...TGA....A.T.G.A......A.....AG.GCG..AAGC.----.AA.....G.TC.
BZ921_10          ....T.T...T...AG...A...TGA....A.T.G.A......A.....AG.GCG..AAGC.----.AA.....G.TC.
BZ913_42          ....T.T...T...AG...A...TGA....A.T.G.A......A.....AG.GCG..AAGC.----.AA.....G.TC.
BZ918_16          ....T.T...T...AG...A...TGA....A.T.G.A......A.....AG.GCG..AAGC.----.AA.....G.TC.
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BZ918_40         .A........T...AG.......T.A.........TA......A.......---------------T......C..TA.
B436_6           .A........T...AG.......T.A.........TA......A.......---------------T......C..TA.
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HLB population complexity: Las/Laf/Lam mixed infections

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BZ918_16          ....T.T...T...AG...A...TGA....A.T.G.A......A.....AG.GCG..AAGC.----.AA.....G.TC.
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HLB population complexity: within species typing using phage sequences

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                  10        20        30        40        50        60
              ....|....|....|....|....|....|....|....|....|....|....|....|....|
B429_FL_12    TGTTAAGCTTCGCAATGACCCAACACCCCGGATAAAAAACCTCCCTCGGATCGCTCACAGGTTGC
B429_FL_14    .................................................................
B429_FL_11    .................................................................
B429_FL_10    .................................................................
B429_FL_9     .................................................................
B429_FL_6     ..C..............................................................
B429_FL_4     .................................................................
B429_FL_2     .................................................................
B429_FL 4     .................................................................
239_TW5       ....G..T..G...................C..................................
239_TW4       ....G..T..G...................C....................T.............
India10       ....G..T..G...................C....................T.............
India17       ....G..T..G...................C....................T.............
India24       ....G..T..G...................C....................T.............
India25       ....G..T..G...................C....................T.............
India3        ....G..T..G...................C....................T.............
India4        ....G..T..G...................C....................T.............
China1_       ....G..T..G...................C....T...............T.............
239_TW1       ....G..T..G...................C....................T.............
239_TW2       ....G..T..G...................C....................T.............
239_TW3       ....G..T..G...................C....................T.............
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Tomimura et al. (2009), Phytopathology 99:1062-1069

Acknowledgements

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Foreign Disease/Weed Science Research Unit (FDWSRU)

Objectives:

1. Deal with newly emerging crop diseases.
2. Prevent the introduction and spread of foreign diseases.