# How a surprising lab result suggests a nutritional means to control HLB.

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CRDF Citrus Research and Development Foundation, Inc.

**The hypothesis:** foliar phosphate fertilization will reduce CLas titer and HLB symptoms.

**The mechanism:** foliar phosphate fertilization will reduce citrate levels in phloem and starve the pathogen.

Discovered by a simple laboratory observation.

The hypothesis is supported by 14 lines of evidence from many labs.

Needs to be rigorously tested.

# Lines of evidence:

1. Citrate as preferred carbon and energy source for *Liberibacter crescens*.
2. Optimal level of citrate needed for *L. crescens* growth known.
3. CLas has very similar metabolism compared to *L. crescens*.
4. Level of citrate in citrus phloem similar to that required for optimal growth of *L. crescens.*
5. Level of citrate in ACP hemolymph similar to that required for optimal growth of *L. crescens*.
6. Level of citrate in citrus leaves increases 2.5-fold with CLas infection.
7. CLas infected psyllids possess nearly 20 times more citrate than uninfected psyllids.
8. Citrate is loaded in phloem in response to P deficiency.
9. Citrate is then exported to roots to solubilize insoluble P.
10. P fertilization is not recommended for Florida citrus growers because of the high insoluble P content of Florida soils. However that P is usually insoluble and can lead to high citrate content in Florida citrus.
11. Infected citrus expresses a small RNA that is associated with P deficiency.
12. Lower P levels in CLas infected leaves compared to uninfected controls.
13. Foliar P fertilization reduced symptoms in a 3-year field trial.
14. Phosphite applications may exacerbate the problem.

# Lines of evidence (only three of these from my lab):

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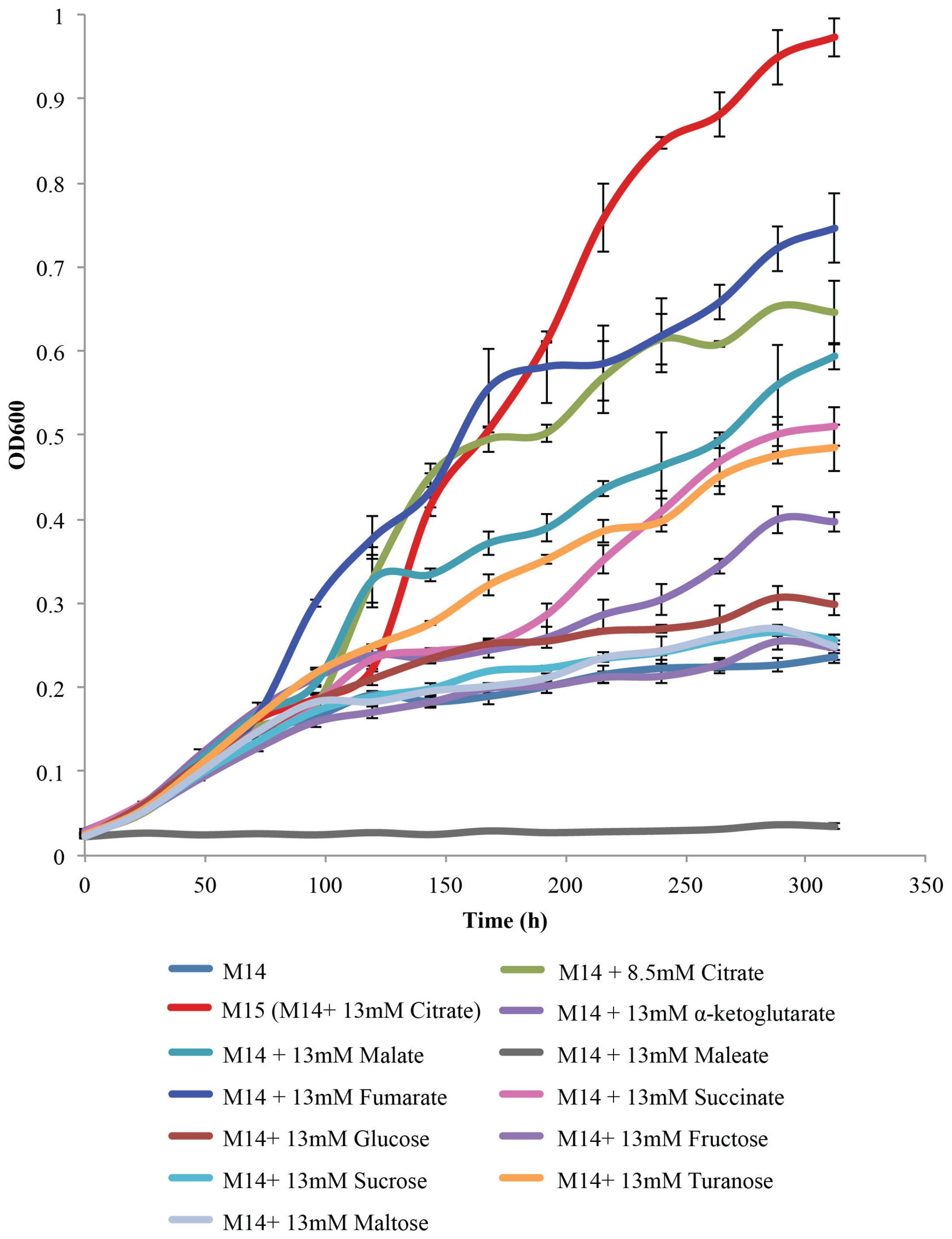
14. Phosphite applications may exacerbate the problem.

# Lines of evidence:

**1. Citrate as preferred carbon and energy source for *Liberibacter crescens*.**

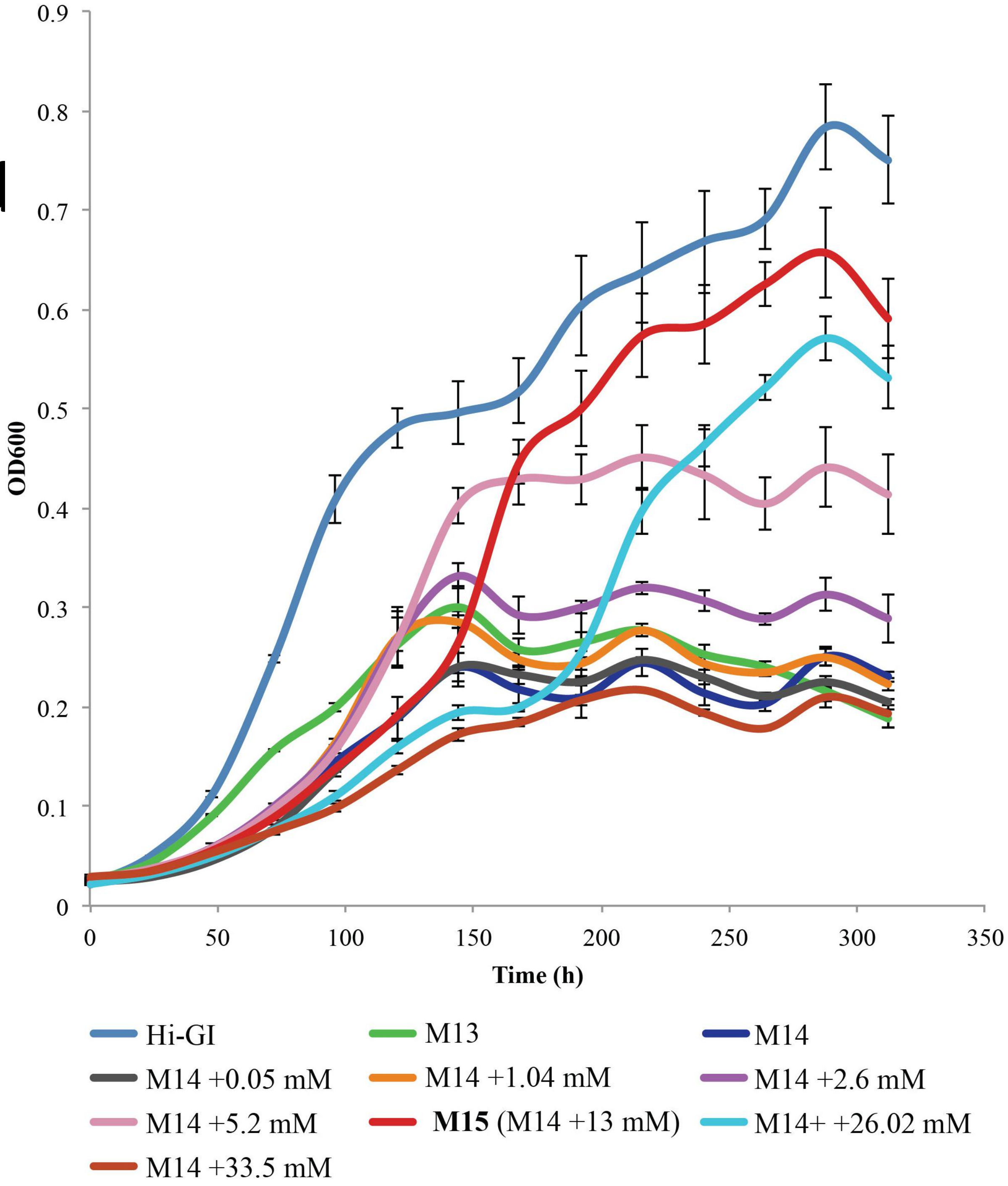
Citrate is preferred over sugars (glucose, sucrose, maltose, fructose, turanose) or other organic acids (malate, fumarate, maleate, succinate, alpha-ketoglutarate).



# Lines of evidence:

**2. Optimal level of citrate needed for *L. crescens* growth known.**

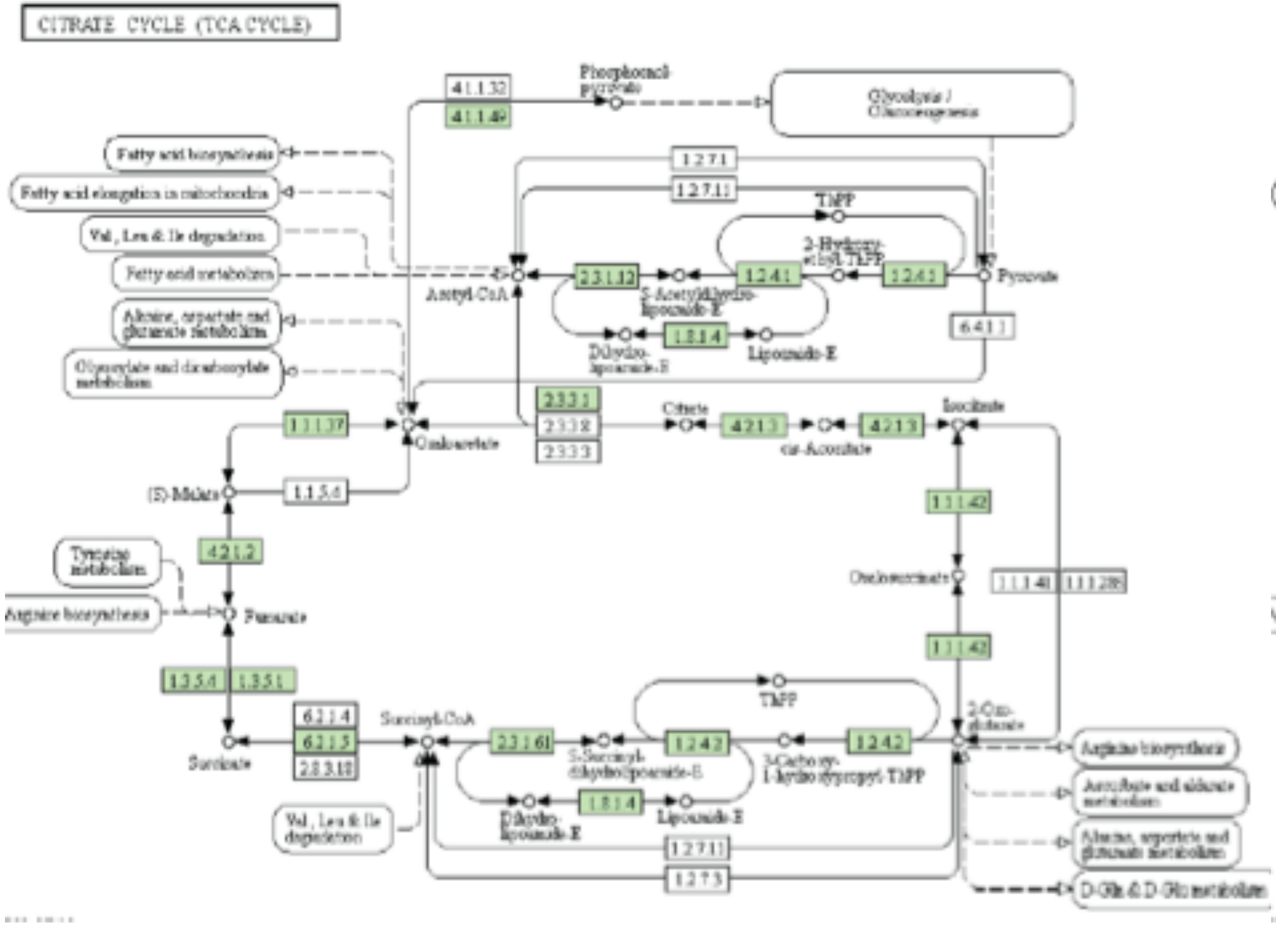
13 mM provided optimal growth i the defined medium.

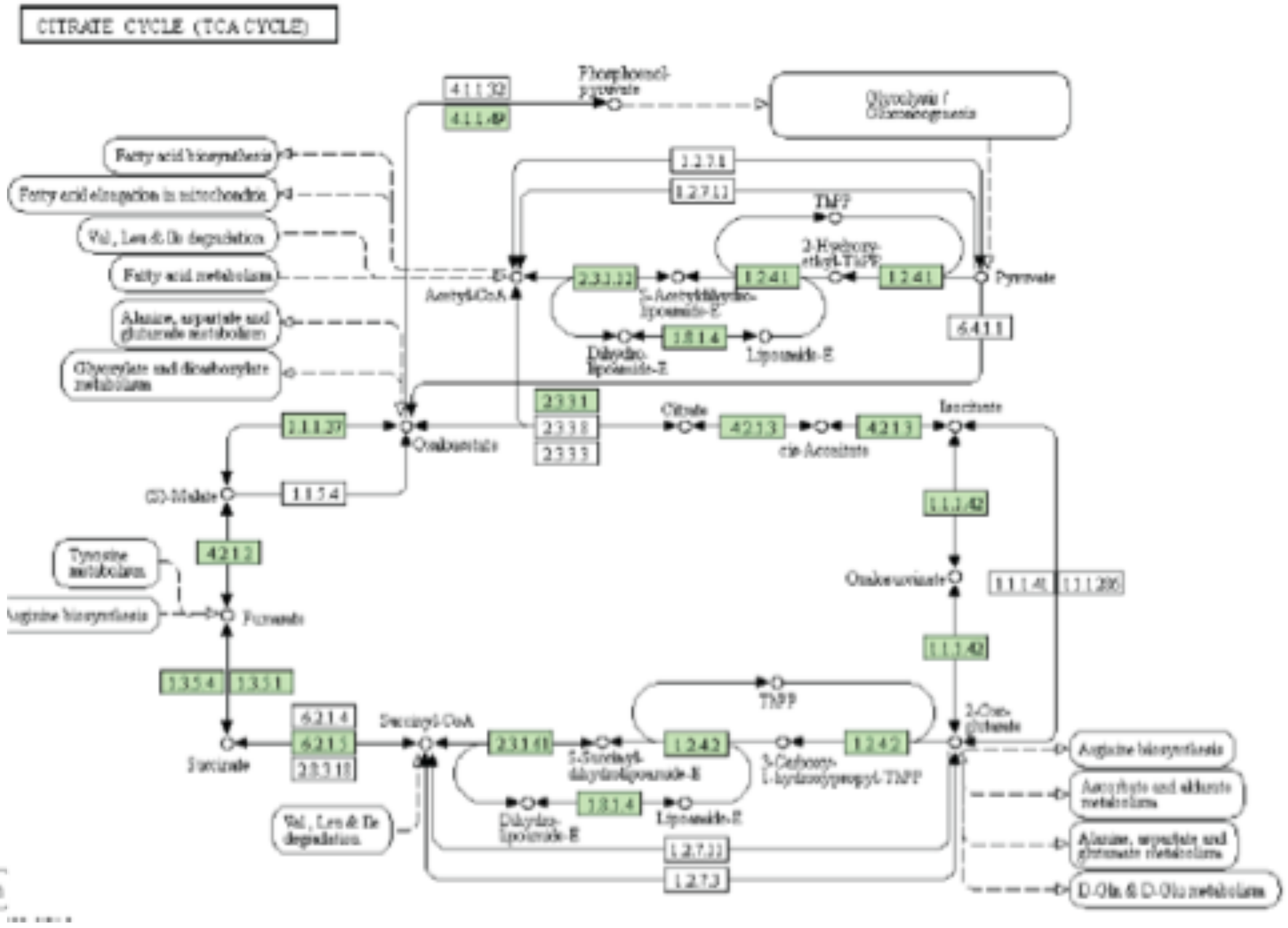


# Lines of evidence:

## 3. CLas has very similar metabolism compared to *L. crescens*.

Example: TCA cycle



# Lines of evidence:

## 4. Level of citrate in citrus phloem similar to that required for optimal growth of *L. crescens*.

Mean [citrate] = 8.5 mM

**Table 3S.** Mean concentrations (*n*=5-7) of organic and fatty acids detected in citrus phloem saps by TMS derivatization and GC-MS. Compounds detected in three or more varieties were quantified (in mM ±SD) or shown as not detected (nd). Varieties are grouped by tolerance to HLB disease with Group 1 being most susceptible and Group 4 most tolerant.

| Variety | Group 1 | | | | | Group 2 | | | | | Group 3 | | Group 4 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | VO | PO | DG | RR | MV | M | ML | PL | SO | VL | SB | CC | PT | CL |
| **Citric acid** | 3.00±1.14 | 6.22±1.74 | 1.04±0.26 | 7.82±1.37 | 5.69±1.15 | 21.4±10.2 | 3.12±0.92 | 5.89±0.94 | 2.74±0.37 | 9.64±3.57 | 33.5±14.5 | 6.66±3.44 | 5.38±3.91 | 4.65±2.22 |
| **Fumaric acid** | 0.18±0.02 | 0.45±0.17 | 0.40±0.14 | 1.02±0.32 | 2.32±0.57 | 1.19±0.27 | 0.31±0.13 | 2.02±0.21 | 0.35±0.19 | 1.31±0.25 | 0.32±0.05 | 0.95±0.85 | 0.41±0.58 | 5.14±0.69 |

Varieties abbreviations: Valencia sweet orange (VO), Pineapple sweet orange (PO), Madam Vinous sweet orange (MV), Duncan grapefruit (DG), Ruby red grapefruit (RR), Sour orange (SO), Volkamer lemon (VL), Alemow (M), Palestine sweet lime (PL), Mexican lime (ML), Carrizo citrange (CC), Severinia buxifolia (SB), Poncirus trifoliata (PT), and Citrus latipes (CL).

Killiny N (2017) Physiol. Molec. Plant Pathol. 97:20-29.

# Lines of evidence:

## 5. Level of citrate in ACP hemolymph similar to that required for optimal growth of *L. crescens*.

**Mean [citrate] = 8.5 mM**

**Same as citrate concentration found in citrus phloem.**

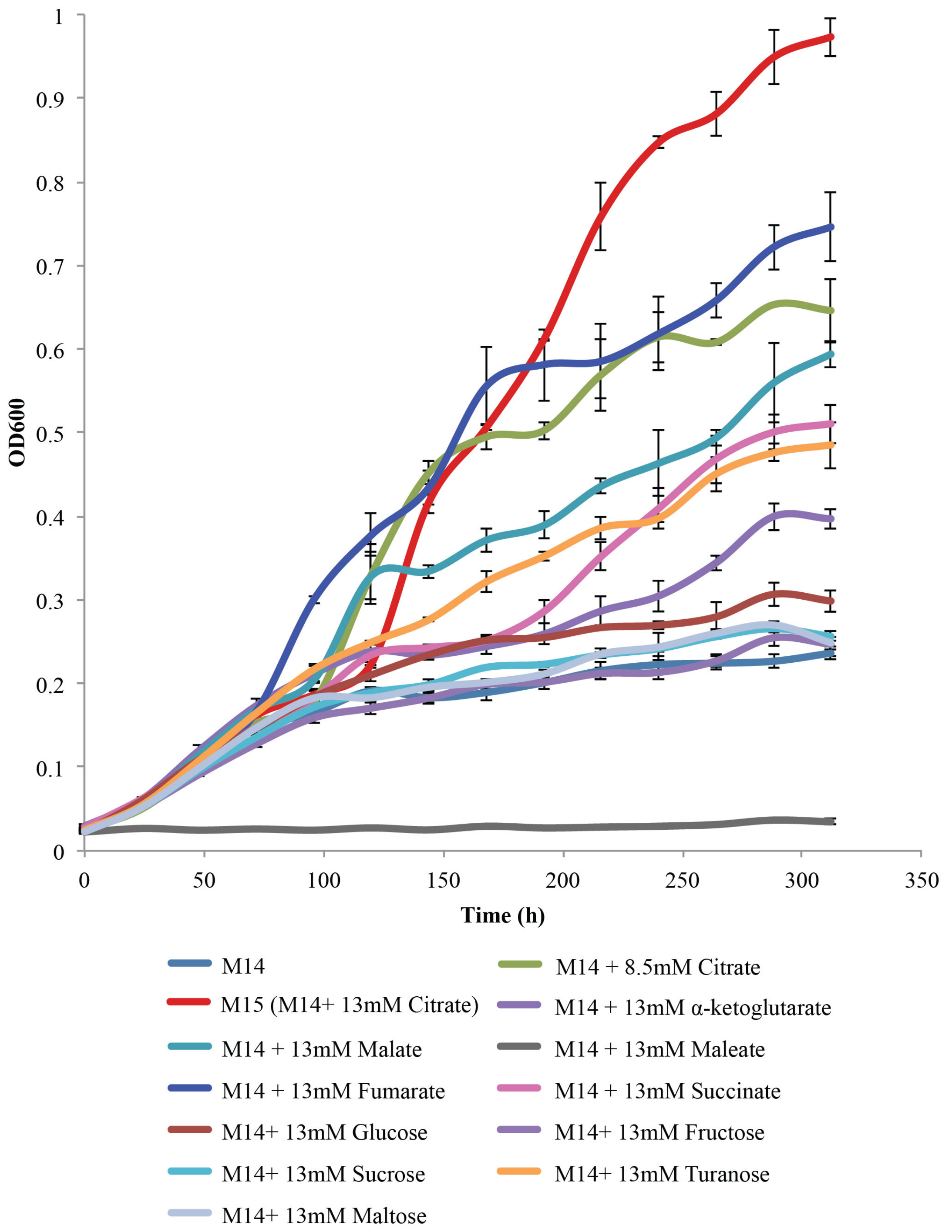
This level supports the growth of ***Liberibacter crescens*** very well.

Calculated from data of Killiny et al. (2017) Virulence 6:1-11.

# Lines of evidence:

*Liberibacter crescens* grows very well at 8.5 mM.

Hence, levels of citrate found in citrus phloem and in the psyllid will support *Liberibacter* growth very well.



# Lines of evidence:

***6.*** **Level of citrate in citrus leaves increases 2.5-fold with CLas infection of Valencia leaves (mg/gFW).**

| | Control | CLas-infected |
|---|---|---|
| citric acid | 3.859 ± 0.396 | 9.574 ± 0.715 |
| fumaric acid | 4.001 ± 0.246 | 6.740 ± 0.733 |
| succinic acid | 7.619 ± 0.340 | 17.431 ± 3.327 |
| malic acid | 39.713 ± 8.163 | 17.937 ± 4.439 |

Killiny & Nehela (2017) MPMI 30:666-678.

# Lines of evidence:

**7. CLas infected psyllids possess nearly 20 times more citrate than uninfected psyllids.**

Only one other compound goes up as much: mannitol (>16x).

Mannitol provides the osmotic environment required by CLas.

Killiny et al. (2017) Virulence 6:1-11.

**Table 1.** Concentrations of different metabolic compounds (mean ± std) detected in Asian citrus psyllid, *D. citri* body after the infection with CLas using GC-MS.

| Compound | Control (*n* = *10*) | CLas-infected (*n* = *40*) | *P*-value | Compound | Control (*n* = *20*) | CLas-infected (*n* = *20*) | *P*-value |
|---|---|---|---|---|---|---|---|
| **Amino acids** (ng insect$^{-1}$) | | | | **Sugars** (ng insect$^{-1}$) | | | |
| L-Alanine | 13.517 ± 6.259 | 24.587 ± 7.540 | 0.0048 | Erthrulose | 0.128 ± 0.039 | 0.201 ± 0.069 | 0.0038 |
| L-Valine | 0.186 ± 0.071 | 0.184 ± 0.063 | 0.9498 | Lyxose | 0.754 ± 0.100 | 1.249 ± 0.212 | 0.0000 |
| Iso-Leucine | 0.487 ± 0.137 | 0.778 ± 0.173 | 0.0015 | Fructose | 4.228 ± 0.337 | 6.079 ± 0.773 | 0.0000 |
| L-Proline | 0.708 ± 0.179 | 0.334 ± 0.093 | 0.0030 | Mannose | 0.028 ± 0.004 | 0.050 ± 0.009 | 0.0000 |
| Glycine | 3.860 ± 1.150 | 5.280 ± 1.625 | 0.0317 | Glocose | 9.129 ± 0.406 | 8.857 ± 1.079 | 0.3341 |
| L-Serine | 1.451 ± 0.170 | 2.401 ± 0.773 | 0.0000 | *β*-Glucopyranose | 0.019 ± 0.002 | 0.021 ± 0.003 | 0.2237 |
| L-Threonine | 1.333 ± 0.513 | 1.933 ± 0.446 | 0.0340 | GlcNAc | 0.129 ± 0.077 | 0.202 ± 0.117 | 0.0889 |
| L-Aspartic acid | 0.357 ± 0.059 | 0.207 ± 0.042 | 0.0009 | Sucrose | 0.124 ± 0.028 | 0.115 ± 0.022 | 0.4772 |
| Glutamic acid | 1.135 ± 0.329 | 1.958 ± 0.564 | 0.0004 | Turanose | 0.094 ± 0.003 | 0.090 ± 0.002 | 0.0553 |
| L-Phenylalanine | 0.163 ± 0.061 | 0.231 ± 0.167 | 0.1207 | Trehalose | 0.091 ± 0.003 | 0.096 ± 0.006 | 0.0093 |
| L-Lysine | 0.673 ± 0.382 | 0.563 ± 0.264 | 0.5284 | Maltose | 0.093 ± 0.003 | 0.096 ± 0.008 | 0.1263 |
| Total | 23.871 ± 6.850 | 38.456 ± 9.327 | 0.0015 | Total | 14.818 ± 0.669 | 17.057 ± 1.853 | 0.0001 |
| **Non-proteinogenic amino acids** (ng insect$^{-1}$) | | | | **Sugars alcohol** (ng insect$^{-1}$) | | | |
| L-Homoserine | 0.047 ± 0.027 | 0.058 ± 0.029 | 0.4146 | Erythritol | 0.386 ± 0.017 | 2.060 ± 0.315 | 0.0000 |
| Pyroglutamic acid | 1.547 ± 0.288 | 0.399 ± 0.162 | 0.0001 | Xylitol | 0.305 ± 0.093 | 0.350 ± 0.065 | 0.3098 |
| GABA | 0.148 ± 0.022 | 0.402 ± 0.136 | 0.0000 | Glucitol | 0.254 ± 0.079 | 0.417 ± 0.199 | 0.0046 |
| Putrescine | 0.868 ± 0.261 | 0.864 ± 0.234 | 0.9766 | Mannitol | 0.524 ± 0.050 | 8.623 ± 0.711 | 0.0000 |
| Total | 2.610 ± 0.492 | 1.723 ± 0.273 | 0.0058 | *Chiro*-Inositol | 0.118 ± 0.017 | 0.352 ± 0.063 | 0.0000 |
| **Organic acids** (ng insect$^{-1}$) | | | | *Scyllo*-inositol | 0.241 ± 0.125 | 0.332 ± 0.107 | 0.1422 |
| Pyruvic acid | 0.006 ± 0.003 | 0.005 ± 0.002 | 0.2618 | *Myo*-Inositol | 3.380 ± 0.579 | 2.996 ± 0.920 | 0.2268 |
| Lactic acid | 0.011 ± 0.004 | 0.007 ± 0.003 | 0.0689 | SA1 | 0.180 ± 0.034 | 0.237 ± 0.078 | 0.0139 |
| Glycolic acid | 0.003 ± 0.001 | 0.003 ± 0.000 | 0.3060 | SA2 | 0.227 ± 0.074 | 0.300 ± 0.107 | 0.0793 |
| Succinic acid | 0.386 ± 0.080 | 0.185 ± 0.050 | 0.0011 | Total | 5.615 ± 0.654 | 15.668 ± 1.451 | 0.0000 |
| Malic acid | 0.910 ± 0.117 | 0.486 ± 0.132 | 0.0000 | **Sugars acids** (ng insect$^{-1}$) | | | |
| Threonic acid | 0.446 ± 0.102 | 0.477 ± 0.106 | 0.5206 | 2-Ketoglutonic acid | 0.103 ± 0.011 | 0.143 ± 0.043 | 0.0003 |
| Citric acid | 0.133 ± 0.035 | 2.633 ± 0.578 | 0.0000 | 2-Ketoglutaric acid | 0.089 ± 0.010 | 0.085 ± 0.011 | 0.4484 |
| Total | 1.895 ± 0.291 | 3.796 ± 0.556 | 0.0000 | Ribonic acid | 0.244 ± 0.166 | 0.319 ± 0.211 | 0.3740 |
| **Fatty acids** (ng insect$^{-1}$) | | | | Gluconic acid | 0.162 ± 0.051 | 0.152 ± 0.041 | 0.6722 |
| Palmitoleic acid | 0.271 ± 0.083 | 0.197 ± 0.090 | 0.0919 | Mannonic acid lactone | 0.108 ± 0.023 | 0.138 ± 0.062 | 0.0663 |
| Palmitic acid | 0.083 ± 0.032 | 0.082 ± 0.035 | 0.9536 | D-Glucuronic acid | 0.055 ± 0.006 | 0.057 ± 0.013 | 0.6598 |
| Oleic acid | 0.251 ± 0.197 | 0.253 ± 0.200 | 0.9793 | Total | 0.760 ± 0.176 | 0.894 ± 0.245 | 0.1554 |
| Total | 0.605 ± 0.283 | 0.532 ± 0.275 | 0.5889 | | | | |
| **Phospho-compounds** (ng insect$^{-1}$) | | | | | | | |
| Erythrose-4- phosphate | 0.398 ± 0.111 | 0.378 ± 0.173 | 0.7318 | | | | |
| Glucose-6-Phosphate | 0.0510 ± 0.0072 | 0.050 ± 0.010 | 0.8524 | | | | |
| Phytic acid | 0.0514 ± 0.0065 | 0.046 ± 0.007 | 0.1327 | | | | |
| Phosphoric acid | 17.913 ± 1.119 | 26.153 ± 1.827 | 0.0000 | | | | |
| Total | 18.414 ± 1.217 | 26.628 ± 1.821 | 0.0000 | | | | |

# Lines of evidence:

## 8. Citrate is loaded in phloem in response to P deficiency.

Lots of support for this in the literature:

A review: Gerke J (2015) J. Plant Nutr. Soil Sci. 178:351-364.

Has been studies since early 1950s: Lipton et al. (1987) Plant Physiol. 85:315-317.

Its summary statement: "P acquisition can be strongly improved by the release of carboxylates and should be taken as a challenge for basic and applied research."

Citrate levels particularly high in calcareous soils.

Calcareous soils common in Florida.

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## 9. Citrate is then exported to roots to solubilize insoluble P.

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**10. P fertilization is not recommended for Florida citrus growers because of the high P content of Florida soils.** However that P is usually insoluble and can to high citrate content in Florida citrus.

Rogers ME, Dwedney MM, Vashisth T, eds. (2017) 2017-2018 Florida Citrus Production Guide. http://www.crec.ifas.ufl.edu/extension/pest/

Zekri M, Obreza T (2013) Phosphorous (P) for citrus trees. EDIS #SL379, http://edis.ifas.ufl.edu/ss581

Reitz HJ (1958) Current recommendations for fertilizing citrus trees in Florida. Proc Florida State Horticul Soc 71:175-179.

# Lines of evidence:

**11. Infected citrus expresses a small RNA that is associated with P deficiency.**

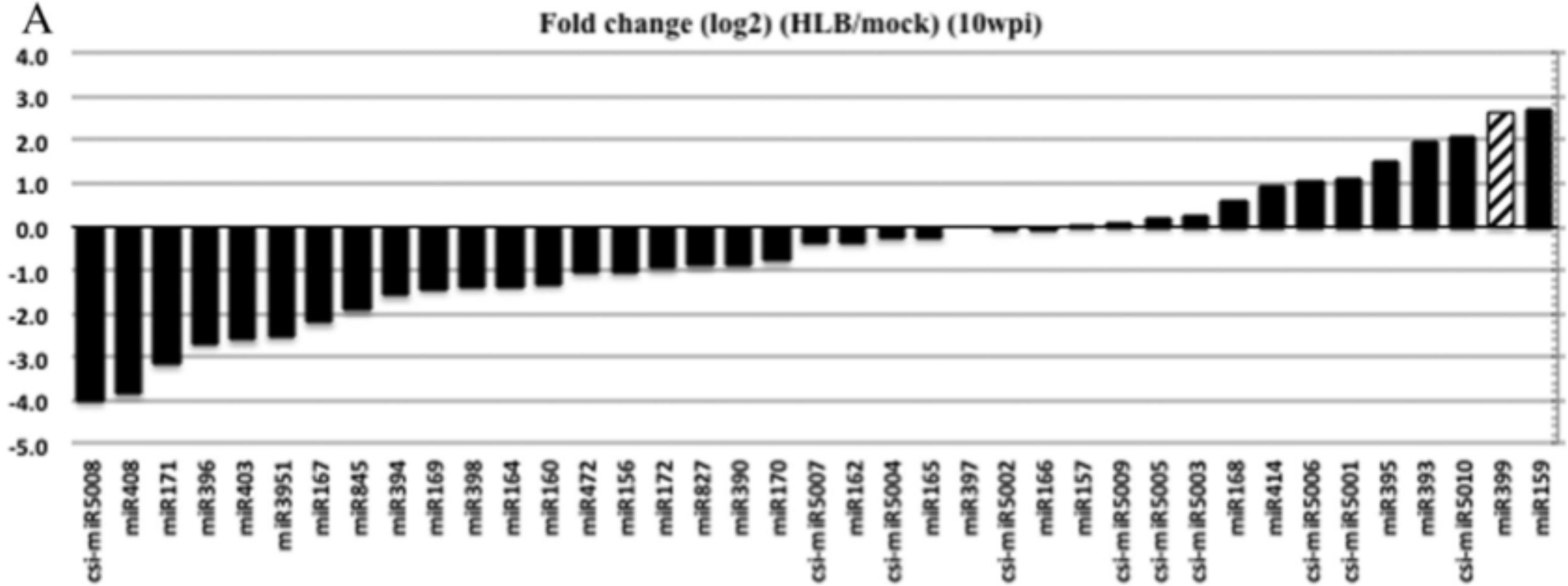



Figure 1A from Zhao et al. (2013) Molecular Plant 6:301-310.

miR399 also induced during P deficiency in Arabidopsis, rapeseed, and pumpkin.

# Lines of evidence:

**12. Lower P levels in CLas infected leaves compared to uninfected controls.**

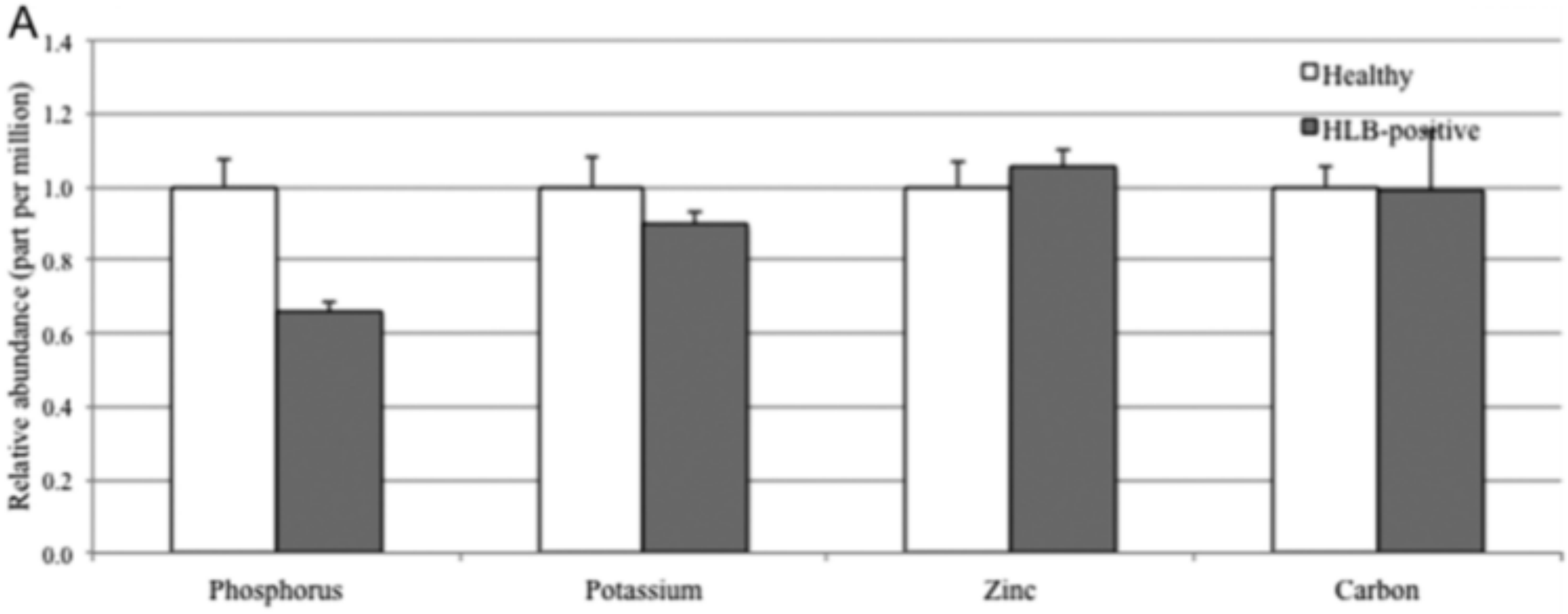



Figure 4A of Zhao et al. (2013) Molecular Plant 6:301-310.

# Lines of evidence:

**13. Foliar P fertilization reduced symptoms in a 3-year field trial in SW Florida.**


Figure 5A of Zhao et al. (2013) Molecular Plant 6:301-310.

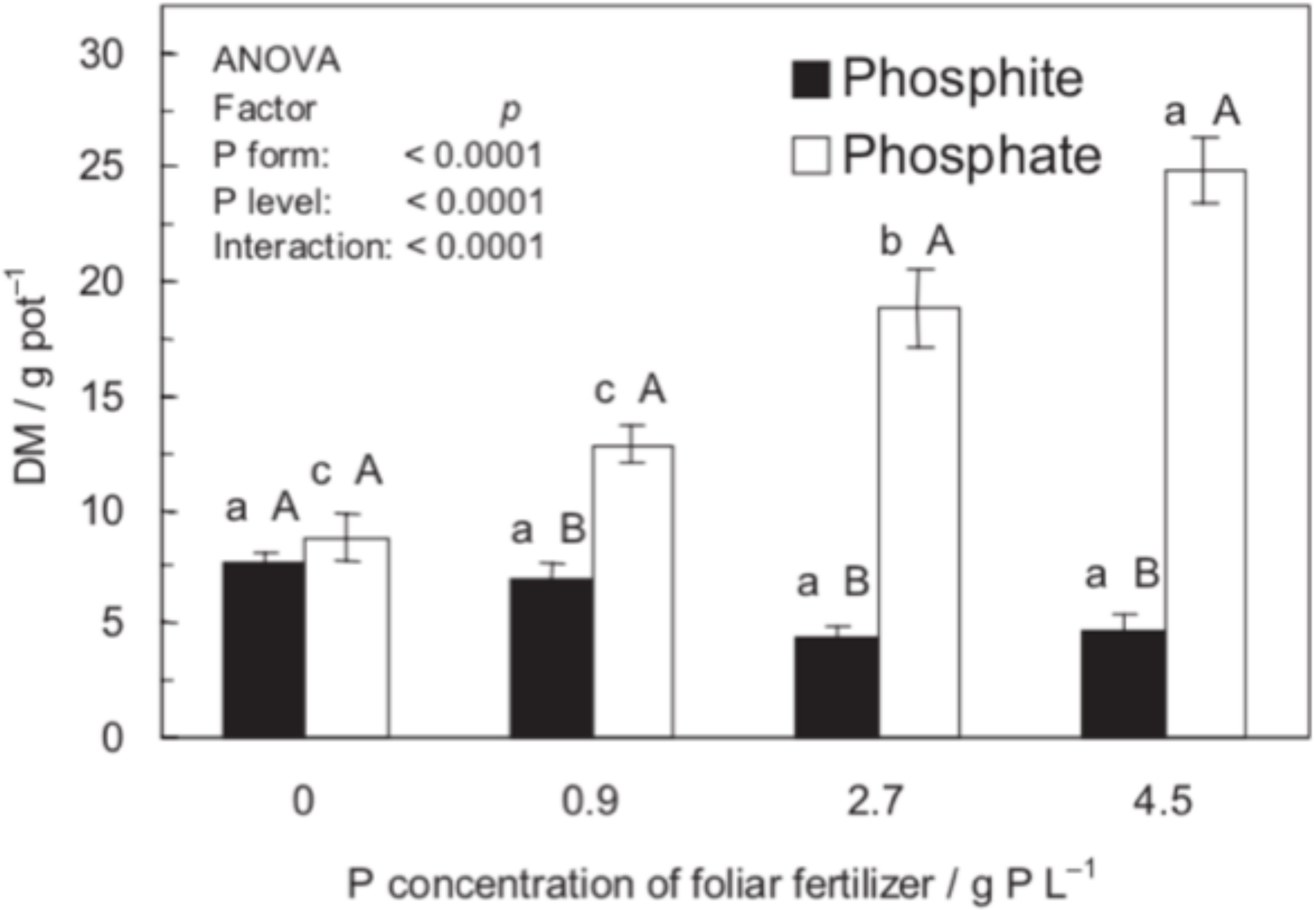
# Lines of evidence:

## 14. Phosphite applications may exacerbate the problem.

**Phosphite is not a source of P for plants.**

Figure 1 of Ratjen & Geáendás (2009) J. Plant Nutr. Soil Sci. 172:821-828.

Phosphite can compete with phosphate for uptake and utilization but with no benefit.



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## 14. Phosphite applications may exacerbate the problem.

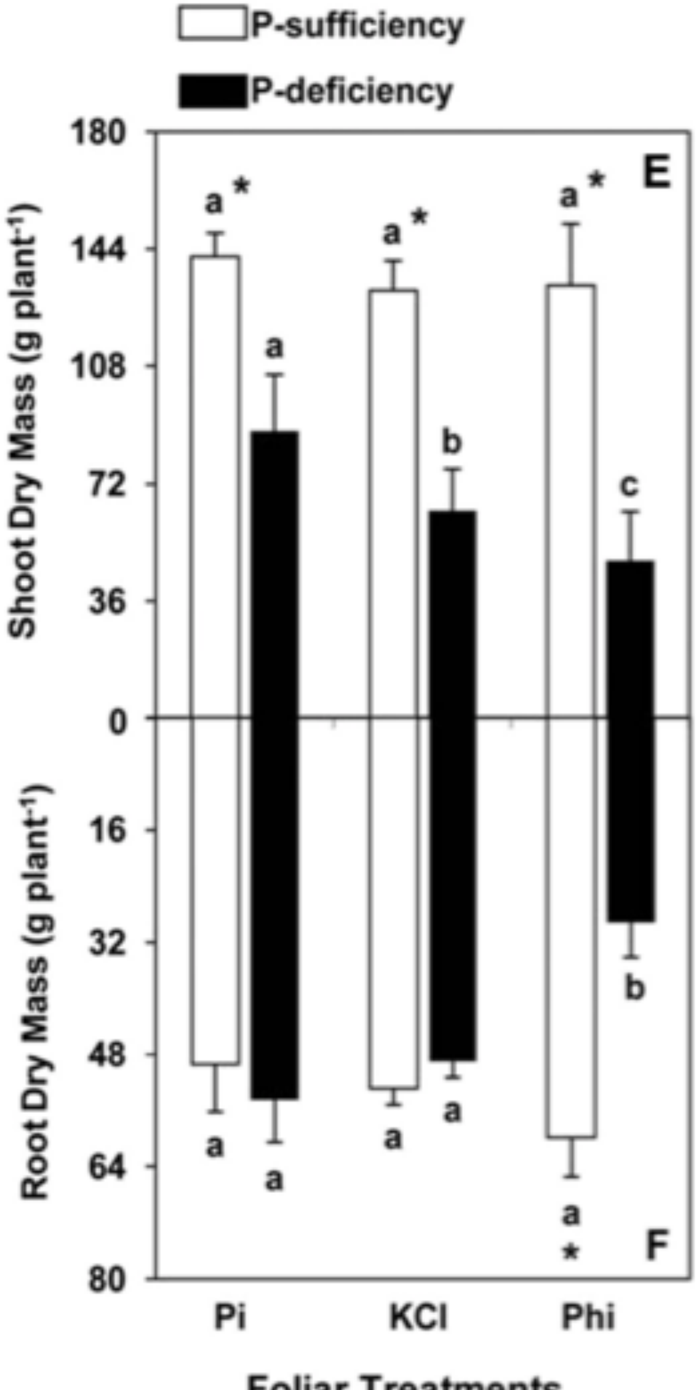
Phosphite makes P deficiency worse in citrus. Roots get larger in attempt to mine more P.

Figure 1 of Zambrosi et al. (2017) 418:557-569.

Phosphite damages the ultrastructure of citrus leaves.

Find other ways to control Brown Rot Disease

– copper, phenylamides?



# Expected consequences of foliar P strategy:

1. No effect on fruit quality expected:

   Phloem citrate is not the source of citrate in fruit – comes from juice sac cells.

2. Foliar P fertilization is not expected to increase P runoff from citrus groves:

   Citrus will no longer need to solubilize insoluble P and stop excreting citrate.

   Citrus will instead use P applied foliarly – only apply that needed to replace P used by fruit and new growth.

   P runoff is expected to decline with this strategy.

   Current solubilization and leaching of insoluble P.

3. Foliar P should reduce CLas titers in citrus phloem and reduce future infection.
4. Will other control measures for CLas still be needed?
5. If phosphites exacerbate HLB, how should brown rot disease be controlled?

   use copper (which may also help control CLas) or phenylamides

   http://ipm.ucanr.edu/PMG/r107100711.html

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# Next steps:

The hypothesis that foliar P fertilization can reduce CLas infection and HLB symptoms through a reduction in phloem citrate levels needs to be rigorously tested.

What level and frequency of foliar P fertilization is required to drive phloem citrate levels below 0.5mM?

Do the phloem citrate levels result in lower CLas infection and HLB symptoms?

Does foliar P fertilization reduce CLas titer psyllids?

Does foliar P fertilization result in lower psyllid fecundity?

If P foliar fertilization works, can we afford to lower our guard on other control measures?

# Solutions to agricultural problems can come unexpected sources.

During the course of our citrate discovery, we were NOT looking for an HLB control method.

*Liberibacter crescens* nutritional requirements was studied to optimize media for CLas.

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**HLB is a tough problem requiring many perspectives.**

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