Efficacy and safety assessment of rhamnolipids for use as a disease suppression agent in citrus trees infected with *Candidatus* Liberibacter asiaticus

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Phase 1: Preliminary experiments (*in vitro*)

Research questions

1a. Can *C*Las be cultured?
1b. What happens when rhamnolipids are applied to a culture of *C*Las?
1c. Development of methods to detect rhamnolipids

Experiments

1a-b. Preparation and testing of *Candidatus* Liberibacter asiaticus (*C*Las) culturing

Media that is capable of cultivating *C*Las will be prepared in accordance with the methodology developed by our collaborators. Due to its limited capacity for aerobic respiration (Lin et al., 2011), *C*Las will be cultured in an anaerobic growth chamber. Bacterial growth will be measured using one or more of the methodologies listed in **Table 1**. The methodology selected will depend on absolute quantities of *C*Las that is able to be cultured and the associated environmental conditions (based on our collaborator's methods). The identity of *C*Las will be confirmed by RT-qPCR (Bastianel et al., 2005), gram staining (Claus, 1992), and genomic sequencing (Zheng et al., 2015).

Table 1. Different methodologies for assessing bacterial growth		
Proliferation and/or Survival	Bacterial growth can be measured by: (1) viable counts of living bacteria *via* colony-forming unit (CFU) counts (2) indirect total population count *via* turbidity (measured using a spectrophotometer) (3) staining assay (4) fluorescent based imaging	**Option 1** requires that *C*Las be culturable on stationary media and is able to survive at low titers. **Option 2** requires that *C*Las be culturable in non-stationary media. **Option 3** is ideal for determining the number of viable cells present in a cell suspension. **Option 4** requires *C*Las to be culturable for several passages (Caldwell et al., 1992).

1c. Development of methods to detect rhamnolipid

Rhamnolipids have been previously characterized by NMR using 1-dimensional (1H, 13C, DEPT-135) and 2-dimensional (COSY, HMQC, HMBC) methods using deuterated methanol as the calibration standard; other methods include Thin-Layer Chromatography, LC-MS, and MS-MS (Sharma et al., 2007). Methods will be developed to detect the presence of this compound in the plant.

Phase 2: Efficacy and validation experiments (greenhouse)

Research questions

2a. What happens to citrus metabolism when rhamnolipids are injected into a citrus tree?

2b. What happens when rhamnolipids are injected into a citrus tree infected with *C*Las?

2c. What happens to other bacteria in the rhizobiome and phytobiome when rhamnolipids are injected into a citrus tree +/- *C*Las

Experiments

2a. Detecting rhamnolipids in citrus plant tissue

Greenhouse-grown citrus trees (n=12) from one variety (orange, lemon, OR mandarin) will be injected with rhamnolipids through the hypocotyl (stem area between the roots and canopy). Trees will be destructively sampled after 1d, 7d, 14d, and 21d post-exposure and leaves, stems, roots, and branches will be collected for analysis. Rhamnolipids and plant metabolites will be extracted and analyzed from plant tissue using methodology developed in our lab.

This experiment will answer the following questions: (1) At what concentration and for how long can rhamnolipids be detected in plant tissue; (2) Once injected, are rhamnolipids detectable in all parts of the plant (except fruit at this stage); and (3) Do rhamnolipids alter metabolism of healthy citrus?

2b-c. Evaluating the *in planta* effect of rhamnolipids on *C*Las-exposed citrus trees

One-year old greenhouse-grown trees (n=40) from one variety of citrus (orange, lemon, OR mandarin) will be grown at the contained research facility (CRF). Trees will be divided into 5 groups: (1) prevention (n=10, *C*Las exposure, rhamnolipid treatment 1 day post-exposure; (2) early-stage disease (n=10, *C*Las exposure followed by rhamnolipid treatment when infection confirmed by the metabolomics early detection method); (3) late-stage disease (n=10, *C*Las exposure followed by rhamnolipid treatment when visual symptoms appear); (4) control (n=5, no *C*Las exposure, rhamnolipids are applied at the start of the experiment); (5) true control (n=5, no *C*Las exposure, no rhamnolipid treatment). Treatment plants will be exposed to *C*Las *via* grafting with infected budwood or direct injection of the pathogen from culture. Control trees will receive grafts from uninfected budwood or direct injection of the vehicle. The visual symptoms of HLB used to classify disease progression will include: curled/deformed leaves, chlorosis, corked veins, mottling, twig dieback, thick/leathery leaves. Samples will be collected according to the schedule outlined in **Table 2**. Leaf samples will be collected for RT-qPCR (monthly), ^{1}H NMR-based metabolomics and/or MS/MS analysis for citrus metabolites and rhamnolipid detection respectively (monthly), and microbiome (every other month for the phytobiome) until plants fruit (approx. 12 months), after which they will be destructively sampled (leaves, hypocotyl, stems, roots, fruits, soil, flowers, bark).

This experiment will answer the following questions: (1) Do rhamnolipids prevent acquisition of *C*Las; (2) At what stage of disease are rhamnolipids most effective (early (before symptom

development), or late (after symptoms appear)); (3) Do rhamnolipids alter metabolism of healthy citrus; (4) How long can rhamnolipids be detected after treatment; and (5) Do rhamnolipids affect the microbiome in the phyllosphere, the rhizosphere, and the soil.

During the experiment, samples will be collected for potential transcriptomic profiling if it is determined that there appears to be significant effects on metabolism assessed through metabolomics profiling to determine what is the cause of the metabolic differences (for example, if rhamnolipids affect plant cell wall integrity). We will also perform experiments to determine if there are changes in electrolyte leakage.

Phase 3: Health and environmental impact

Research questions

3a. Do rhamnolipids affect microbial communities in the gut?
3b. What happens to metabolism and the gut microbiome of mice fed rhamnolipids that are administered at a concentration comparable to that (if detected) in fruit, or what might be ingested in the form of juice?
3c. What happens to ACP if they are carrying *C*Las in their gut (do rhamnolipids affect transmissibility of *C*Las from ACP)
3d. What happens to ACP that are feeding on a tree treated with rhamnolipid
3e. What happens to other (beneficial) insects exposed to rhamnolipid (for example, could rhamnolipid affect bee microbiome / bee health)?

Proposed study timeline

	2018				2019				2020			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Phase 1: Preliminary experiments (*in vitro*)												
Material Acquisition: relevant materials will be sent from Brazil to California			■									
1a. Preparation and plating of *Candidatus* Liberibacter asiaticus (*C*Las) onto media				■								
1b. Incubation of *C*Las with rhamnolipids					■							
1c. Characterization of rhamnolipid for detection in plants etc.					■	■						
Data analysis						■						
Phase 2: Efficacy and validation experiments (greenhouse)												
2a. Detecting rhamnolipids in citrus plant tissue			▨	▨								

2b-c. Evaluating the *in planta* effect of rhamnolipids on *C*Las-exposed citrus trees		■	■	■	■							
Data Analysis		■	■	■	■	■						
Manuscript Preparation												
Phase 2								■	■	■		

Once Phase 1 is completed, we will work on defining the experiments for phase 3 of the study. We propose that the project be completed in 3 phases. Phase 1 will serve to replicate the results from Brazil in our hands. Phase 2 will begin at the same time to show that rhamnolipids are effective at killing *C*Las *in planta*. Toward the end of phase 2, we will begin phase 3 to assess the safety in humans / animals / the environment. We envision that many manuscripts will be generated from these results. These studies will test: (1) the effectiveness of rhamnolipids in treating citrus infected by HLB, (2) the impact on the health and safety of humans or animals consuming fruit from a tree treated with rhamnolipids; (3) whether rhamnolipids change the rhizosphere, phyllosphere, and soil microorganisms of citrus trees; (4) whether rhamnolipids pose a threat to beneficial insects; and (5) potential impacts on ACP and their ability to carry the bacterium.

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