Asymptomatic spread of huanglongbing and implications for disease control

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Huanglongbing (HLB) is a bacterial infection of citrus trees transmitted by the Asian citrus psyllid Diaphorina citri. Mitigation of HLB has focused on spraying of insecticides to reduce the psyllid population and removal of trees when they first show symptoms of the disease. These interventions have been only marginally effective, because symptoms of HLB do not appear on leaves for months to years after initial infection. Limited knowledge about disease spread during the asymptomatic phase is exemplified by the heretofore unknown length of time from initial infection of newly developing cluster of young leaves, called flush, by adult psyllids until the flush become infectious. We present experimental evidence showing that young flush become infectious within 15 d after receiving an inoculum of Candidatus Liberibacter asiaticus (bacteria). Using this critical fact, we specify a microsimulation latent period transmission model that they clearly influence the initial spread of HLB and the distribution of symptomatic trees on the longer time scale of 1–2.5 y or, as recently documented, 6 y (6). Spatially explicit modeling, however, is incorporated in a recent study of HLB epidemic outbreaks (2). Although our specific focus and the comments above pertain to HLB, it is important to note that there is a considerable literature using spatially explicit models of transmission of plant pathogens incorporating both compartment and stochastic compartmental models, where systems of ordinary differential equations represent the dynamics (2–5). The geometry of groves and the foci of psyllid entry generally are not taken into consideration in these models, despite the fact that they clearly influence the initial spread of HLB and the distribution of symptomatic trees on the longer time scale of 1–2.5 y or, as recently documented, 6 y (6). Spatially explicit modeling, however, is incorporated in a recent study of HLB epidemic outbreaks (2). Although our specific focus and the comments above pertain to HLB, it is important to note that there is a considerable literature using spatially explicit models of transmission of plant pathogens incorporating both compartment models (7) (SI Appendix, refs. 10–14) and agent-based models (8, 9).

Significance

Huanglongbing (HLB) is a vector-transmitted bacterial infection of citrus trees that poses a major threat to the citrus industry in Florida, Texas, and California. Current control strategies that focus on the vector, the Asian citrus psyllid Diaphorina citri, are usually initiated when the trees become symptomatic, anywhere from 10 mo to several years after initial infection. We show, experimentally, that newly infected young leaves can become infectious within 10–15 d after receiving an inoculum of bacteria from an adult psyllid. We then show by microsimulation of the asymptomatic spread of HLB through a grove under different invasion scenarios and control strategies that reduction of up to 75% of adult psyllids and nymphs can enhance citrus production.

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Two forms of delays are important for the modeling of the spread of HLB across a grove. These delays are as follows: (i) the time from initial infection of young flush on a tree until the onset of disease symptoms and (ii) the time from initial infection of young flush by adult psyllids until the flush become infectious. An experimental study shows a minimum time to symptoms of \( \sim 200 \) d and a latent period bounded above by \( 60 \) d (1).

The purposes of this paper are as follows: (i) to present new experimental evidence on the elapsed time from initial infection of young flush until the flush become infectious to previously uninfected adult psyllids and to nymphs; (ii) to describe an agent-based transmission model that can mechanistically produce the spatial patterns of rapid proliferation of \( Ca. \) Las infection and its intensity in response to a variety of spatially explicit psyllid invasion scenarios in groves of previously uninfected citrus trees, emphasizing the asymptomatic period; and (iii) to demonstrate, via modeling, the potential impact of surveillance and intervention strategies applied while trees are asymptomatic and focused on reduction of adult psyllids, as well as nymph stages, in the psyllid life cycle.

**Time to Infectiousness of CitrusFlush Following an Initial Infection by Psyllids**

**Experimental Infections.** Psyllids were obtained from an existing psyllid colony that originated in Florida citrus groves. The colony is currently maintained in growth rooms on source plants containing \( Ca. \) Las originally inoculated in 2007 from symptomatic plant samples that were taken from locations where nymphs developed. As nymph stages, in the psyllid life cycle.

| Experiment no. | No. of input psyllids | No. of plants/no. of with nymphs | \% Ca. Las\(^+\) | % Ca. Las\(^+\) | No. of plants positive (total)\(^a\) |
|---------------|-----------------------|---------------------------------|----------------|----------------|-----------------|
| 1             | 50/6/2                | 25\(^a\)                        | 38 \( \leq 30 \) d | 2/2 \( \leq 30 \) d |
| 2             | 50/6/2                | 21\(^a\) (33\(^b\))            | 0 \( \leq 30 \) d | 0/2 \( \leq 30 \) d |
| 3             | 50/6/2                | 33\(^a\)                        | 33 \( \leq 30 \) d | 1/2 \( \leq 30 \) d |
| 4             | 50/6/6                | 24\(^a\)                        | 5 \( \leq 30 \) d  | 2/4 \( \leq 10 \) d |
| 5             | 50/6/2                | 55\(^a\)                        | 5 \( \leq 30 \) d  | 2/2 \( \leq 33 \) d |
| 6             | 50/6/2                | 69\(^a\)                        | 13 \( \leq 31 \) d | 1/2 \( \leq 33 \) d |
| 7             | 50/6/1                | 29\(^a\)                        | 8 \( \leq 31 \) d  | 1/1 \( \leq 31 \) d |
| 8             | 50/6/1                | 38\(^a\)                        | 13 \( \leq 31 \) d | 1/1 \( \leq 31 \) d |
| 9             | 50/4/2                | 54\(^a\)                        | 22 \( \leq 31 \) d | 1/2 \( \leq 30 \) d |
| 10            | 50/4/2                | 100\(^a\)                       | 54 \( \leq 30 \) d | 2/2 \( \leq 30 \) d |
| 11            | 100/10/6              | 51\(^a\)                        | 16 \( \leq 30 \) d | 4/6 \( \leq 32 \) d |
| 12            | 50/6/nd               | 5\(^a\) (21\(^b\))             | 12 \( \leq 30 \) d | nd              |
| 13            | 50/6/nd               | 17\(^a\) (21\(^b\))            | 0 \( \leq 22 \) d  | nd              |
| 14            | 50/6/nd               | 8\(^b\) (21\(^b\))             | 17 \( \leq 22 \) d | nd              |
| 15\(^d\)      | nd/6/2                | nd                             | 83 \( \leq 30 \) d | 2/2 \( \leq 38 \) d |
| 16\(^d\)      | nd/3/3                | nd                             | 29 \( \leq 30 \) d | 3/3 \( \leq 58 \) d |

\( nd, \) not determined.

\(^a\)quantitative PCR analysis of Ca. Las in infected areas of plants that harbored eggs and nymphs before \( 30 \) d.

\(^b\)Percentage of Ca. Las-positivepsyllids in the population on HLB-infected plants from which the input nymphs were removed.

\(^c\)Percentage of Ca. Las-positive input nymphs withdrawn from the cages at \( 15 \) d.

\(^d\)Experiments used psyllid progeny from earlier experiments.
control and the necessity of surveillance of psyllid populations in asymptomatic groves.

Microsimulation Modeling of Transmission. We focus on movement of *Ca.* Las between psyllids and patches of flush as the units of analysis, ignoring within-tree consequences of infection. This decision prioritizes young flush as the sites of initial infection on trees and their role as infectious agents. *Ca.* Las can move from tree to tree via migration of infected adult psyllids. We introduce the day as the time step for transmission activities. For the vectors, we keep track of age and infection status (infected or not) of individual adult psyllids, eggs, and the five instar stages, which we label nymphs. For young flush, we keep track of their age since emergence, their occupancy by eggs and nymphs, and their infection status (uninfected, infected but not infectious, and infectious). In the model, a grid of trees (or a set of flush patches) is an $11 \times 25$ lattice with integer coordinates $c=(c_1, c_2)$. Although flush patches are evenly spaced in this formal specification, the close spacing of trees within a row and the wide between-row spacing in a real grove (SI Appendix, Fig. S1) are taken into account by our assuming much higher probabilities of within-row relative to between-row migration of adult psyllids. We assume that there are three flushing seasons: spring (60 d at 25 °C), summer (30 d at 28 °C), and fall (15 d at 25 °C).

We summarize the vector and flush development processes and the accompanying transmission dynamics in our model by describing the activities that take place on a typical day during a flush season. A full algorithmic description of the system dynamics is presented in SI Appendix. There are six steps that occur on a flush patch located at a generic site with coordinates $c=(c_1, c_2)$. In their order of execution, these steps are (i) emergence of new flush shoots, (ii) migration of adult psyllids to new flush patches, (iii) psyllid aging and mortality, (iv) egg laying and egg survival, (v) *Ca.* Las transmission from infectious flush to nymphs, and (vi) *Ca.* Las transmission from adult psyllids to flush. Parameters specified by a numerical value followed by a second value in parentheses are temperature-dependent and correspond to $25^\circ C$ (and $28^\circ C$). Parameters used in the simulation model are given in Table 2:

### i) Emergence: Each day during a flushing period, 20 new flush, unoccupied by psyllids, emerge at $c$. They join cohorts of 20 flush each that emerged on previous days in the flushing period. Flush from previous days may be occupied by eggs, nymphs, and adults, each identified by an infection status. Young flush are regarded as such for 13 (16 at 28 °C) d, after which they are transferred to the category “old,” along with their resident psyllid populations.

### ii) Migration: Adult psyllids at $c$ each have a probability of 0.4 of migrating on the given day. Conditional on being selected to migrate, a psyllid at $c$ in the interior of the grove can move to $(c_1 \pm 1, c_2)$ with a probability of 0.025 for each option or to $(c_1, c_2 \pm 1)$ with a probability of 0.475 for each option. Psyllids from each of the above four destinations can, correspondingly, migrate to $c$ according to the same rules operating at their points of origin. In-migrants distribute among flush by selecting a shoot with probability $s_0$ of surviving to the next day, Nymphs that survive for 13 (11 at 28 °C) consecutive days after initiating nymph status emerge as adult psyllids. Emerging adults on the given day have probability $s_a$ of surviving to the next day, at which time they can take part in *Ca.* Las transmission to flush if they are infected. Adult psyllids already present in the flush patch are also selected with probability $s_b$ for survival and participation in the transmission process (step vi) below on the given day. The survivors are labeled according to gender, infection status, and flush shoot identifier. Nymphs are labeled by flush shoot identifier and infection status.

### iii) Psyllid aging and mortality: Each nymph has probability $s_c$ of surviving to the next day. Nymphs that survive for 13 (11 at 28 °C) consecutive days after initiating nymph status emerge as adult psyllids. Emerging adults on the given day have

### Table 2. Parameters used in simulation model

| Parameter description | Value | Units | Source |
|-----------------------|-------|-------|--------|
| Maximum flush age     | 30    | d     | (15)   |
| Flush shoots emerging  | 20    | d −1  | Calculated* |
| Egg-to-adult transition| 17 (14)  | d     | (16, 17) |
| Duration of young flush| 13 (16)  | d     | Calculated |
| Proportion of migrating adults | 0.4  | d −1 | Assessed |
| Between-row probability | 0.95 |       | Assessed |
| Egg duration          | 4 (3)  | d     | (17)   |
| Nymph duration        | 13 (11)  | d     | (17)   |
| Daily flush shoot capacity | 40   | Eggs | Calculated* |
| Eggs laid per female adult | 10   | Eggs | (18)   |
| Transmission from flush to nymphs | 0.083 | d −1 | Assessed* |
| Transmission from adult to flush | 0.3  | d −1 | Assessed |
| Latent period         | 10–21 | d     | (17, 20) |
| Egg/nymph survival probability | 0.8614 (0.8343)  | d −1 | (19) |
| Adult survival probability | 0.9847 (0.9659) | d −1 | (17, 20)* |
| Nymph infection age    | 9 (8)  | d     | (17, 21) |

*Calculation found in section demographic parameter values.

*Values represent simulation parameters for $25^\circ C$ ($28^\circ C$).

*Values assessed from experiments in Table 1 (details are provided in SI Appendix).
The above steps are repeated on each day during a flushing season, with appropriate modifications for the first 12 (15 at 28 °C) d, when the population of young flush is growing by 20 shoots per day, and there is no transfer of flush to the old category (algorithmic details are provided in SI Appendix).

**Initial Conditions.** For an initially uninfected grove, we start simulation of the transmission process by placing 200 psyllids on either a few trees in a corner of the grove or on selected trees along an edge of the grove. The latter specification is consistent with considerable evidence about how new waves of psyllids arrive at a grove, frequently driven in by the wind. Hall and Hentz (23) used sticky traps to capture psyllid movement in and out of groves during arbitrary times in the year, with a peak time being in the spring. Boina et al. (24) documented movement in both directions between managed and unmanaged groves. We assume that ~30% of the initial psyllid population is infected. Newly arrived female psyllids initiate egg laying, and both male and female psyllids feed on flush. These initial conditions initiate the dynamics described above.

**Demographic Parameter Values.** Daily survival probabilities of the egg and nymphal stages of psyllids are based on field experiments. The percentage of first-instar nymphs that survive to adulthood (19) is 7.91%. There is no field estimate for the survival of eggs, so we assume their survival is the same as the nymph stages to account for the high survival rate found in laboratories and the predation that occurs in the field. The daily survival rates for eggs and nymphs at 25 °C is (0.0791) 1/17 = 0.8614, where 17 is the number of days after which the transition from egg to adult (16) occurs. The adult psyllid daily survival probability was estimated using a t(1/2) of 45 d via (1/2) 1/45 = 0.9847 (17, 20). In addition, in field observations, we find, on average, 100 eggs per flush shoot. It is assumed that eggs become nymph stages to account for the high survival rate found in laboratories and the predation that occurs in the field. Field evidence indicates that 30,000 eggs per flush yields 1,200 flush accumulating over 60 d. Thus, the number of new flush per day, Nf, should be Nf = 20.

**Results**

Six sets of invasion conditions are introduced to convey the variation in rate of spread of infection consequential to them. These sets are as follows: (i) 200 psyllids, 30% of which are infected, are placed on four trees in the southwest corner of the grove; (ii) condition i AND 35% of randomly selected trees are occupied by 200 uninfected psyllids; (iii) 200 psyllids, 30% of which are infected, are placed on six trees on the southern edge of the grove and on 11 trees on the eastern edge of the grove; (iv) condition ii AND 35% of randomly selected trees are occupied by 200 psyllids, none of which are infected; (v) 10 trees distributed around the center of the grove are occupied by 200 psyllids, 30% of which are infected; and (vi) condition v AND 35% of randomly selected trees from the remaining 265 sites are occupied by 200 psyllids, none of which are infected. It is assumed that the time from initial infection of a young flush until it becomes infectious is 15 d.

The central point of the results in Table 3 is that not only the number of trees containing infected psyllids at the start of an invasion but the number of trees initially occupied by uninfected psyllids influences the time to infection of a full grove. Invasion scenarios i–iv are four of a myriad of possible initial conditions where a given grove is invaded by psyllids from an adjacent grove in a large multigrove system (6). Scenarios v and vi correspond to two of many possible initial distributions of psyllids that may be blown into a grove by the wind (26). The variation in time until 100% of the trees are infected as shown in Table 1 is undoubtedly an underestimation relative to field conditions in the absence of controls, because multiple psyllid invasions over time are an important feature of the introduction of HLb to a grove. We are entirely lacking empirical investigations of invasion processes in working citrus groves, to say nothing of controlled introductions over time in experimental groves.

**Table 3. Elapsed time (days) from initial invasion until 100% of the trees are infected in the absence of control measures**

| Invasion condition | Mean time until 100% of trees infected |
|--------------------|---------------------------------------|
| (i) Corner         | 781 ± 18*                             |
| (ii) Corner + 35%  | 450 ± 41*                             |
| (iii) Edge         | 424 ± 14*                             |
| (iv) Edge + 35%    | 234 ± 57*                             |
| (v) Middle         | 400 ± 8*                              |
| (vi) Middle + 35%  | 169 ± 11*                             |

*95% confidence interval based on 50 simulation runs.

![Fig. 1](image.png)
analyses of variable spread rates in replicated invasions described by Melbourne and Hastings (27) are relevant to our problem, and suggest a pressing need for experimental studies of psyllid invasions and attempts to monitor such invasions in natural settings. A more extensive discussion of variable spread rates consequential to psyllid invasions is given in SI Appendix.

Fig. 1 shows the advance of infection and the variation of intensities through the grove under scenario iii, where infection is introduced along the southern and eastern edges of the grove. We show the results of a strategy where either reduction in adult psyllids or reduction in both adults and nymphs occurs on days 16 and 30 of each flushing period. Here, it is important to emphasize that elimination of adult psyllids without harming nymphs is hypothetical, not corresponding to the use of a known pesticide. There are, however, pesticides that act on nymphs only, as well as pesticides that act on the combination of adults and nymphs. Here, it is assumed that 75% of both adult psyllids and nymphs are eliminated at each attack in Fig. 1B, whereas only 75% of adult psyllids are eliminated at each attack in Fig. 1C. The sharp contrast between Fig. 1B and Fig. 1C shows the major role of transmission from infected flush to nymphs, and to emerging adults from them. Failure to control this transmission link has serious consequences for the spread of infection. Overall, there is considerable reduction in the fraction of infected psyllids relative to the intense infection picture (Fig. L4) in the absence of control measures.

The number of psyllids on an individual tree varies over time as indicated in Fig. 2. Peak numbers of psyllids are present after the start of each flushing period. For all panels, black lines represent the total adult psyllids, blue lines represent healthy adult psyllids, and red lines represent infected adult psyllids. No control (A), elimination of 75% of adult psyllids and nymphs on days 16 and 30 of each flushing period (B), and elimination of 75% of adult psyllids on days 16 and 30 of each flushing period (C) are shown. The green segments on the horizontal axis (i.e., days since initial invasion of the grove) correspond to flushing periods.

We show the results of a strategy where either reduction in adult psyllids or reduction in both adults and nymphs occurs on days 16 and 30 of each flushing period; (ii) without effective control measures, occupancy of many trees by uninfected psyllids at the start of an invasion promotes the spread of infection throughout the grove. A more fine-grained representation of each invasion x intervention scenario, analogous to Figs. 1 and 2, is shown in SI Appendix.

**Discussion**

We have shown experimentally that the latency period from new infection by infected adult psyllids to infectiousness in young flush is less than 15 d. In subsequent experiments, most of the plants that were colonized by psyllids developed HLB symptoms, but those plants that did not have nymphs usually failed to develop disease. These intriguing observations have not been quantified and deserve further study. Using the latency period information in a model where feeding by infected adults on young flush subsequently infects nymphs already on the flush, we showed that entire groves can become infected in a few months. The resulting infected trees can all be asymptomatic, and can become home to on the order of 12,000 psyllids, a large fraction of which are infected, during a single flush period. Because trees do not tend to show symptoms for anywhere from 1–2.5 y, and possibly longer, after initially becoming infected, emphasis must be placed on ongoing surveillance and control of psyllids. The invasion scenarios we consider have the common feature that there is a single invasion occurring on a given day, and all transmission of infection and growth of the psyllid population is consequential to these initial conditions. The psyllid counts on the order of 20,000–30,000 observed on single trees in field settings are a consequence of more intensive invasions that take place over time. For example, the edge invasion scenario followed a few weeks later by a middle-field invasion of psyllids would increase the rate of spread of infection and considerably increase the psyllid count for individual trees.

**Table 4.** Proportion of grove occupied by psyllids (top value) and number of trees out of 275 with more than 10% of the occupying psyllids infected (bottom value) on days 151 and 241 under different invasion i-vi and intervention a-d conditions

| Invasion condition | (a) Days 16 and 30* | (b) Days 2 and 30* | (c) Days 16 and 30* | (d) Days 2 and 30* |
|--------------------|---------------------|---------------------|---------------------|---------------------|
| (i) Corner         | 0.324               | 0.407               | 0.251               | 0.287               |
|                    | 0.207               | 0.233               | 0.218               | 0.215               |
| (ii) Corner        | 1.00                | 1.00                | 1.00                | 1.00                |
|                    | 1.00                | 1.00                | 1.00                | 1.00                |
| (iii) Edge         | 0.829               | 0.902               | 0.720               | 0.782               |
|                    | 0.724               | 0.716               | 0.695               | 0.716               |
| (iv) Edge          | 6.48                | 1.00                | 2.00                | 1.00                |
|                    | 1.00                | 1.00                | 1.00                | 1.00                |
| (v) Middle         | 0.844               | 0.938               | 0.702               | 0.793               |
|                    | 0.702               | 0.622               | 0.640               | 0.618               |
| (vi) Middle        | 1.00                | 1.00                | 1.00                | 1.00                |
|                    | 1.00                | 1.00                | 1.00                | 1.00                |

*Days after the start of each flush period.*
fruit free of HLB for 2 + y beyond the time of symptom onset in uncontrolled groves. Such reductions from spraying adult psyllids have been demonstrated in Brazil, for example (28).

The question of how to reach 90% reductions in psyllid populations relative to uncontrolled conditions in a multigrove setting is starting to get serious consideration from grower cooperatives (www.crec.ifas.ufl.edu/extension/chmas/chma_overview.shtml). A critical first step is synchronization of insecticide spraying schedules on a regional basis to reduce movement of psyllids drastically from one grove to the next, as well as to reduce their prevalence within groves. In addition, the use of aluminized mulch (29) to protect newly planted trees for the roughly 2 y it takes before a canopy prevents effective utilization of this methodology has the potential to delay the introduction of psyllids to a grove during this period of early development. Other psyllid control tools are under development and suggest that the stringent targets we have indicated for delaying the onset of symptoms should be within reach.

Psyllid flush transmission is the dominant mode of dispersal of Ca. Las in a grove, as demonstrated by the documented large numbers of psyllids that occupy trees in infected groves, as well as the multiple routes of rapid propagation of this bacterium between psyllids and flush. Better surveillance tools are needed to help quantify the progression of new infection in previously uninfected groves. A start on incidence estimation for an epidemic when it is first discovered and the design of early detection monitoring have been put forth recently (30). However, as the intrinsic asymptomatic transmission illustrated herein indicates, much more needs to be done in this direction.

Materials and Methods

In the experimental study, two plant growth rooms (15 × 19 feet) were prepared with a 14-h photoperiod of photosynthetically active electromagnet radiation (Em) of ~200 μEm−2s−1 using Sylvania T5 fluorescent lamps maintained at ~26 °C. In addition to C. macrophylla, Carissa cittergo (C. Citroncirus weberri) plants were studied at ~6–12 mo of age and were grown from seed. Further, Murraya exotica L. ("orange jasmine") and Bergera koenigii L. plants were obtained externally and also included in the study. Results for the latter three plants are shown in SI Appendix. We include them because they are part of the HLB transmission system on a regional basis, particularly for initial introductions of introduced psyllids to citrus groves. However, it is the within-grove transmission of HLB among asymptomatic trees that is the focus of our modeling exercise, rather than the vastly larger spatial scales involved in intraorchard spread. All plants were pesticide-free at the time of experiments. Plants were caged in 24 × 24 × 36-foot collapsible observation and rearing cages and were continuously maintained within the controlled environment growth rooms.

qPCR reactions were carried out using an ABI 7500 (Applied Biosystems) real-time PCR instrument per the manufacturer’s instructions using HLBaspR primer/probe sets plus a plant COX-based primer probe set as indicated by Li et al. (31). In the analysis of noninfected plants or psyllids, qPCR often results in a cycle threshold (Ct) value in the 30s. Ct values under 30 were considered confidently positive, but higher numbers were considered ambiguous. We ran all ambiguous samples by conventional PCR and called samples positive that resulted in a distinct band in the correct position (11).

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