Let loose the wasps on the orange groves

Insect is used to eliminate transmitter of greening, one of the worst diseases of citrus

Marcos de Oliveira

Solutions are finally coming into sight for the cruelest disease in Brazilian citriculture. The affliction, which goes by the complicated Chinese name *huanglongbing*, or simply “greening” in English, has already proven more aggressive than other diseases of orange groves including citrus canker and citrus variegated chlorosis. New ways of fighting it are more than welcome – right now, the recommended course of action is to uproot the tree and repeatedly douse the area with insecticides. Roughly 14 million orange trees were eradicated between 2005 and 2011.

The most recent solution to contain this agricultural epidemic focuses on fighting the transmitter of *Liberibacter*, the bacterium that causes citrus greening. Populations of the vector insect, a psyllid (*Diaphorina citri*), can be diminished by ecological means, using a wasp, *Tamarixia radiata*, which is harmless to humans and agriculture. These wasps infest young psyllids – while they are still in their flightless nymphal stage – by laying eggs in their bodies. When the wasps hatch, they destroy their hosts. The reproductive cycles of *Tamarixia* and the psyllid were determined by a team led by professor José Roberto Postali Parra, from the Luiz de Queiroz School of Agriculture at the University of São Paulo (Esalq/USP). During studies conducted in the city of Araras (state of São Paulo), the wasps released into local orchards eliminated 51% to 72% of all psyllid nymphs.

The technique was fully mastered in 2011, through studies carried out by the Esalq team of 10 entomology researchers, in a project funded by the Citriculture Defense Fund (Fundecitrus), an entity maintained by citrus growers. To keep the experiment going, Esalq currently produces a total of 60 thousand to 100 thousand wasps every month. They are released into the field in areas with large populations of *Diaphorina citri*, at a proportion of 400 wasps per hectare.

The problem with this type of management, as observed in preliminary studies by Professor Parra – who has also established partnerships with the Campinas Institute of Agronomy (IAC), the Biology Institute of São Paulo, and the University of California, Davis –, is that the wasp migrates into areas where chemicals are being applied to control the disease. And there it dies. So while the practice of massive insecticide spraying persists in commercial orchards, the wasps are being released in areas planted with orange jessamine (*Murraya paniculata*), an ornamental plant used in hedges, belonging to the same family as citrus and which can also host the disease. Additional releases are being made in abandoned orchards and organic citrus groves.

“The results of the research make it possible to recommend a psyllid control strategy involving the ap-

HENRIQUE SANTOS/FUNDECITRUS

The afflicted orange tree has yellowed leaves and fruit that don’t ripen

The psyllid, the insect that transmits citrus greening, was reported in Brazil for the first time in the 1940s

plication of insecticides only during the vegetative rest period of citrus plants (period of lower metabolic activity, in which the plant loses leaves)," says Parra. This technique makes it possible to also release *Tamarixia radiata* in insecticide-treated areas, thereby supplementing the effect of the chemicals.

A series of studies on alternative options for eliminating the psyllid are still in their initial stages. "One of them is the use of bacteria that interfere with the insect's behavior and biology. There are also fungi that can be used as control agents," says Parra. This latter type of biological control is applied similarly to industrial insecticides. The fungi are mixed with water, then sprayed over the insects and groves. The fungus is harmless to plants and humans, but infests both the nymphal and adult stages of the pest insect, drying them out and essentially mummifying them. Professor Parra's research team is examining the possibility of isolating the sexual pheromones of psyllids – substances secreted by the females in order to attract males –, which could be used as traps to diminish the pest's populations.

Guava trees may be the key to another possible solution for stopping the advance of the psyllid. "They produce volatile substances that repel the insect, as was initially observed in Vietnam, where guava and orange trees are planted in alternating patterns within the same orchards," says the agronomist José Belasque Júnior, a researcher at Fundecitrus. The research is currently at the stage where the psyllid repellent compounds are being chemically identified. Studies to identify and synthesize these volatile guava substances are being conducted by the National Institute of Science and Technology (INCT) of Semiochemicals in Agriculture, funded by FAPESP and by the Ministry of Science, Technology and Innovation. The semiochemicals branch of the INCT is based at Esalq and is coordinated by Professor Parra. "The idea is to have the orange trees themselves produce these substances in the future, using transgenics, in order to repel the insect," he explains.

Bacteria and fungi are some of the options for eliminating the insect

Huanglongbing (HLB) was reported in Brazil for the first time in 2004, by researchers at the Sylvio Moreira Citriculture Center (a subsidiary of IAC) and at Fundecitrus. The disease's rapid expansion is visible in an experiment conducted by the research team led by agronomist Marcos Machado, a director at the Citriculture Center, in a project funded by FAPESP between 2005 and 2008 and conducted in partnership with Fundecitrus. The project studied the diagnosis and biology of the greening bacterium, as well as ways to fight it. Fundecitrus researcher Renato Bassanezi isolated a newly planted orange orchard in Araraquara, containing 10 thousand HLB-free plants, surrounded by sugarcane plantations and located at least one kilometer away from any other orchard.

Insecticide-based chemical control was applied, using different types of applications. After three years, 15% of the trees were infected with greening. The conclusion was that, even with intense chemical control, it was not possible to prevent the entry of contaminated insects from other areas. "It's not a simple situation. It might be that relatively few insects invaded the area, but even if only one was contaminated, it would be enough to transmit the disease," says Machado. By

2009, the experimental orchard had been completely decimated by HLB.

The Chinese name *huanglongbing* is translatable as yellow dragon disease or yellow stem disease, as it yellows the leaves and turns the fruit green, deformed, and unfit for either consumption or industrial processing. "The infection is severe. Cutting off branches does no good. You have to pull out the tree, roots and all, with a machine, to make sure that it won't sprout back," says Machado.

There are currently about 160 million citrus trees in the state of São Paulo, each expected to produce for up to 20 years. In 2011, according to Fundecitrus, greening was present in 53.38% of all citrus stands (each having an average of 2,000 trees) in São Paulo state. Other important diseases, such as variegated chlorosis and citrus canker, affected 40.3% and 0.99% of all citrus trees, respectively. HLB is also present in the states of Minas Gerais and Paraná. Together, these three states account for almost 90% of citrus production in Brazil and 60% of the world's frozen juice concentrate, the industry's most important product, which earned it US$2 billion in exports in 2010.

The disease was first reported in Asia – the birthplace of citrus – in the 19th century. It was first described in China and later received the English moniker "greening" in South Africa, in a reference to the fruits that don't ripen (in other words, that stay green). According to Machado, HLB may have arrived in Brazil by means of vegetative propagation materials, over ten years ago. The insect that disseminates the bacteria is an old acquaintance of Brazilian farmers. The psyllid was reported in Brazil for the first time in the early 1940s, and was probably introduced through infested seedlings. The insect adapted well to the local climate, but was not considered a pest because it did not cause any damage, although it had already been reported as a transmitter of the HLB-causing bacterium in China and other Asian countries. Brazilian citrus growers did not change their view on *Diaphorina citri*, a tiny creature just 2 to 3 millimeters long, until HLB was confirmed in São Paulo. The psyllid acquires and transmits the bacteria from diseased trees when it pricks and feeds from their phloem vessels, i.e. their sap circulation system.

PHOTOS HENRIQUE SANTOS/FUNDECITRUS

The disease deforms the fruit and leaves it unfit for either industrial processing or *in natura* consumption

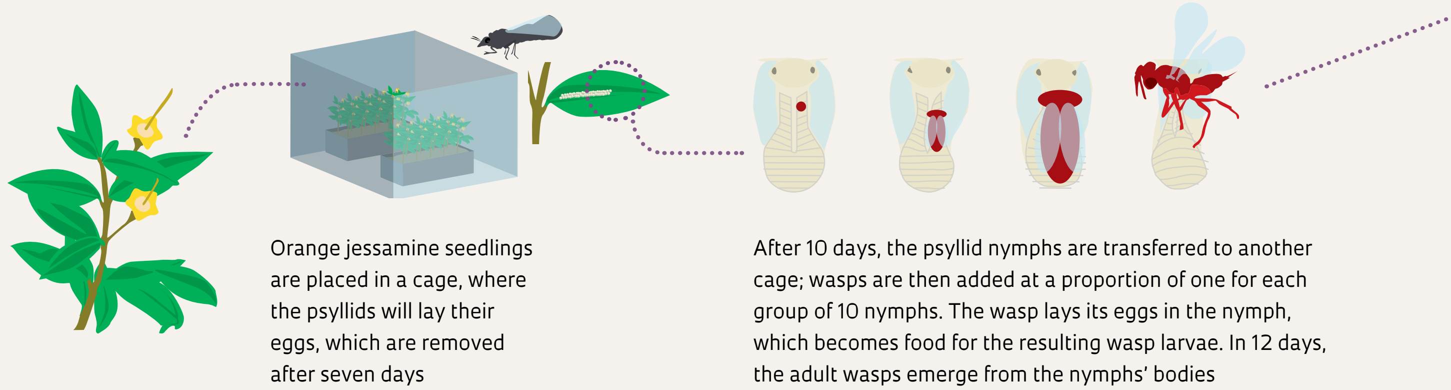
The importance of this vector in the study of the disease soon mobilized Professor Parra, who submitted a project about the insect to FAPESP in 2004. "Up until that time, the insect had not been studied in depth. Its population levels did not justify specific studies and stricter control by growers," he says. "With the thematic project, we were able to learn more about *Diaphorina* and suggest biological and behavioral measures, as well as recommend the rational use of insecticides without throwing the environment out of balance and without killing the psyllid's natural enemies, such as some small wasps," he explains. "We discovered that the insect develops best on other trees, particularly the orange jessamine."

Psyllid females lay eggs on sprouts of those plants. On citrus, they lay an average of 160 eggs, whereas on other plants the number can be as high as 348. "We established climate and zoning parameters covering the areas where the pest occurs most intensely. The greatest prevalence is in the cities of São Carlos, Bariri, Botucatu, Lins, and Araraquara." Professor Parra has been researching agriculture-related insects for over 40 years and feels that understanding and fighting citrus greening is a major challenge, perhaps the biggest in his career. "The insect is difficult to raise in the laboratory. There's also the problem of populations that vary over time, over seasons, and across temperature and rainfall conditions. This has kept us from modeling its presence in the field," he says.

If the insect is complicated, then it makes a good match for *Liberibacter*. The bacterium was identified in 1970 in a laboratory in France, by Joseph Bové's group at the French National Institute of Agronomic Research. To date, it has not been taxonomically identified or received a definitive scientific name of its own, on account of the difficulties in cultivating it with laboratory culture media. It is therefore called *Candidatus Liberibacter (Ca.L.)*, and three species have been

Wasps to battle the psyllid

USP grows *Tamarixia radiata* in the laboratory



associated with HLB: *Ca. L. asiaticus*, responsible for upwards of 90% of the disease in Brazil and the cause of the most destructive infection; *Ca. L. africanus*, milder-mannered and absent from Brazilian orchards; and *Ca. L. americanus*, found only rarely in this country.

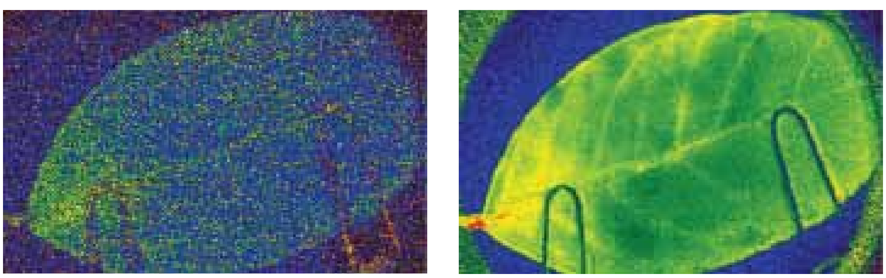
"If you want *Liberibacter* to grow, you need to make a broth to its liking, and this is achieved through a series of trials and errors," says Elliot Kitajima, an Esalq professor and electronic microscopy expert. Kitajima and Francisco Tanaka, also a professor at the same university, produced images of *Liberibacter* in phloem of Madagascar periwinkle (*Catharanthus roseus*), an ornamental plant used as an alternative host of the bacterium. "The concentration in orange trees is very low; it's not possible to produce images like those obtained from periwinkle," he says. "There is no correlation between number of bacteria and damage to phloem," says Machado. Even so, a few bacteria are apparently more than enough to secrete toxins that compromise the phloem's functionality. "Very quickly, about half an hour after the bacteria-carrying insect pricks the plant, it becomes infected, but disease evolution is slow and the symptoms may take up to a year to manifest," Parra says.

In the future, the battle against HLB will also rely on knowledge of the *Liberibacter* genome. The genetic sequencing of *Ca.Liberibacter asiaticus* was completed in 2008 by the U.S. Department of Agriculture. The Asian species has a short genome, about 1.2 million base pairs long, whereas *Xylella fastidiosa*, the bacterium that causes variegated chlorosis, has 2.4 million base pairs and *Xanthomonas axonopodis citri*, the bacterium behind citrus canker, sports 4.5 million.

The shorter genome of *Liberibacter* means that it is even more specialized than the others. "It may indicate that the bacterium is an obligatory parasite of the plant because it is incapable of living on its own," says Machado. The agronomist also coordinates the recently created National Institute of Science and Technology (INCT) of Genomics for Citrus Improvement, which includes institutes and universities in the Brazilian states of São Paulo, Bahia and Paraíba, as well as Florida, in the United States. That American state is also a victim of citrus greening, which was first reported there in 2005. With its more than 60 million orange trees, Florida is the world's vice-champion of orange production, second only to the Brazilian state of São Paulo, which accounts for almost 78% of all oranges produced in Brazil. Together, Florida and São Paulo account for roughly 80% of the world's orange juice production.

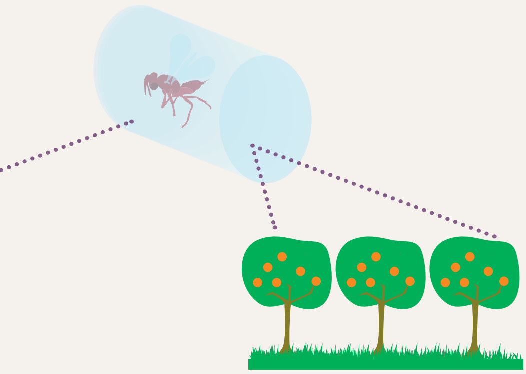
The orange growers are the ones who have to combat the disease. In Brazil, a federal law requires them to eliminate the sick trees, but that's not always what actually happens. "Half of all citrus growers, especially the small ones, don't spray their orchards with insecticides. Though easy, the procedure is not cheap," says Armando Bergamin Filho, a professor at Esalq/USP and coordinator of another FAPESP-funded project on citrus greening, initiated in 2008. "Control must be regional. If a grower applies insecticides and his neighbor doesn't do the same, it will do no good."

Bergamin believes that the disease is controllable, so long as growers across an extended region agree to collaborate to uproot the afflicted trees, spray their orchards, and inspect them – preferably on a monthly basis. In his project, Bergamin



Digital photographs of a greening-infected leaf placed under LED lights, revealing the alterations captured by fluorescence

PHOTOS LUÍS GUSTAVO MARCASSA/IFSC-USP **INFOGRAPHIC** TIAGO CIRILLO



The wasps are then released into the orange groves. In order to reproduce, they lay their eggs in the psyllid nymphs, eliminating them

SOURCE: JOSÉ ROBERTO PARRA AND ALEXANDRE JOSÉ FERREIRA DINIZ/ESALQ

and his group, which includes researchers from Fundecitrus, studied the dissemination of citrus greening over time and the speed with which the infection reaches groves. He also investigated the vector insect, studying the flight habits of the psyllid, which can be carried by the wind over hundreds of meters. The study was entirely based on molecular analyses covering the several stages of the disease.

LED LIGHTED LEAVES

One of the problems faced by agriculturists is identifying HLB by its symptoms, which are very similar to those of other citrus diseases. A more effective and reliable inspection method may soon become available, as shown by the ongoing research on photonic detection systems, conducted by two research groups in São Carlos. The experiments are based on fluorescence imaging, using the light emitted by a leaf after being illuminated by a light emitting diode (LED) or a laser.

One of these studies is being conducted by Luís Gustavo Marcassa, a professor with the São Carlos Institute of Physics at USP, proceeding from a prior study in which the researchers used lasers to identify citrus canker (*see Pesquisa FAPESP* Issue No. 80). "For citrus greening, I am not using a laser, as it requires greater care and is more expensive; instead, I am using high potency LEDs. When we analyze the leaves, we achieve 90% accuracy in identifying the disease in the samples, confirmed through molecular analysis," says Marcassa.

The study consists in illuminating the leaf with a LED and photographing the resulting fluorescence, which is altered by the presence of HLB bacteria. The data are sent to a computer, which plots a graph revealing whether the plant might be infected. Aside from São Paulo, Marcassa, in a partnership with Reza Ehsani, a professor with the Citrus Research and Education Center (Crec) at the University of Florida, also conducted studies in orange groves in Florida. "But the tests in Florida produced different results and, there, the accuracy rate was 61%. Our colleagues in the United States believe that the difference is due to the environmental conditions of the plants, such as fertilizers and nutrients, which are different from those in São Paulo," says Marcassa.

The second experiment is being conducted by researcher Débora Milori, at the Agricultural Instrumentation division of the Brazilian Agricultural Research Corporation (Embrapa) in São Carlos, which studies the use of laser beams and LEDs for early diagnosis of HLB. Milori and her team have invented a portable device that shines a beam of light on the leaves and can diagnose greening and variegated chlorosis with 95% accuracy. In the case of HLB, the equipment can obtain a positive diagnosis even while the plant is still at the asymptomatic stage. The patent on the system is pending in Brazil and internationally, and was licensed in 2011 to the company Opto Eletrônica, also from São Carlos, which has been developing the first prototype for use in the field, in partnership with the researchers at Embrapa.

"Today, visual inspection can lead to 30% to 60% inaccuracy, including confusion with other diseases that present similar symptoms," says Milori. "In the laboratory, with the device calibrated specifically for each variety of citrus, the accuracy levels are high and the results come out in a few seconds. The present challenge is testing it in the field." The study is supported by the National Council on Scientific and Technological Development (CNPq) and by the São Carlos Center for Optics and Photonics Research, one of FAPESP's Research, Innovation and Dissemination Centers. ■

PROJECTS

1. *Bioecology and the establishment of strategies to control Diaphorina citri Kuwayama (Hemiptera: Psyllidae), the vector of the bacteria that causes citrus greening* – No. 2004/14215-0 (2005-2009)
2. *Studies of the bacterium Candidatus Liberibacter spp., causal agent of the citrus huanglongbing disease (formerly greening): diagnosis, biology and management* – No. 2005/00718-2 (2005-2010)
3. *Molecular epidemiology and integrated management of huanglongbing (Asian and American) in the State of São Paulo* – No. 2007/55013-9 (2007-2012)
4. São Carlos Center for Optics and Photonics Research (subproject: *Optics applied to agriculture and to the environment* – No. 1998/14270-8 (2000-2012)
5. *Fluorescence imaging applied to citrus diseases in the field* – No. 2010/16536-9 (2010-2012)

GRANT MECHANISMS

1. to 3. Thematic Project
4. Research, Innovation and Dissemination Center (Cepid)
5. Regular Research Fellowship Award

COORDINATORS

1. José Roberto Postali Parra (USP)
2. Marcos Antonio Machado (IAC)
3. Armando Bergamin Filho (USP)
4. Débora Milori – Embrapa
5. Luís Gustavo Marcassa (USP)

INVESTMENT

1. R$462,875.46
2. R$1,418,367.25
3. R$1,175,226.06
4. R$38,622,748.13
5. R$85,260.51

SCIENTIFIC ARTICLE

Bassanezi, R.B. *et al.* Epidemiologia do *huanglongbing* e suas implicações para manejo da doença. **Citrus Research & Technology.** v.31, n.1, p. 11-23, 2010.

FROM OUR ARCHIVES

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