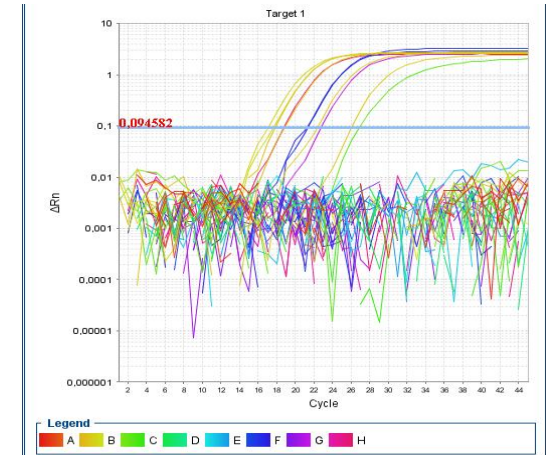


THREAT OF HUANGLONGBING IN THE MEDITERRANEAN REGION: SURVEYS AND ANALYSES OF '*Candidatus Liberibacter*' SPECIES IN PLANTS AND IN *Trioza erytreae*



F. Siverio¹, E. Bertolini², G. Teresani², A. Arilla², L. Peris³, M. Guillén³, O. López³, V. Dalmau³, J.M. Guitián⁴, J.M. Bové⁵, M. Cambra², M.M. López²

¹ ICIA. Canary Islands, Spain. ² IVIA. Valencia, Spain. E-mail: mlopez@ivia.es.
³ GVA. Valencia, Spain. ⁴ Tragsatec. Madrid, Spain. ⁵ INRA and Université Victor Ségalen, Bordeaux, France.

**HUANGLONGBING (HLB): yellow
shoot disease (China)**

GREENING: in fruits (South Africa)

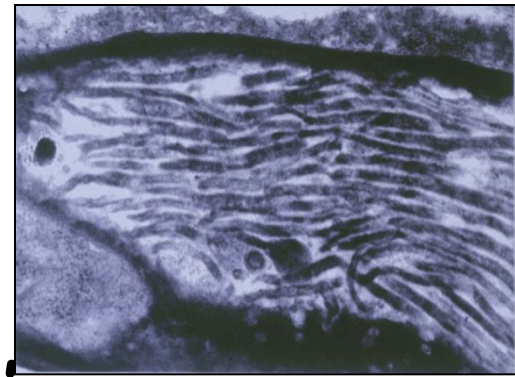


IMPORTANCE OF HLB

- More than 65 million affected citrus trees in Asia, Africa and America
- Limiting factor of the citrus culture
- Very susceptible: sweet orange and mandarin
- High economic impact
- Quarantine organism in the UE and the EPPO, not present in Mediterranean countries

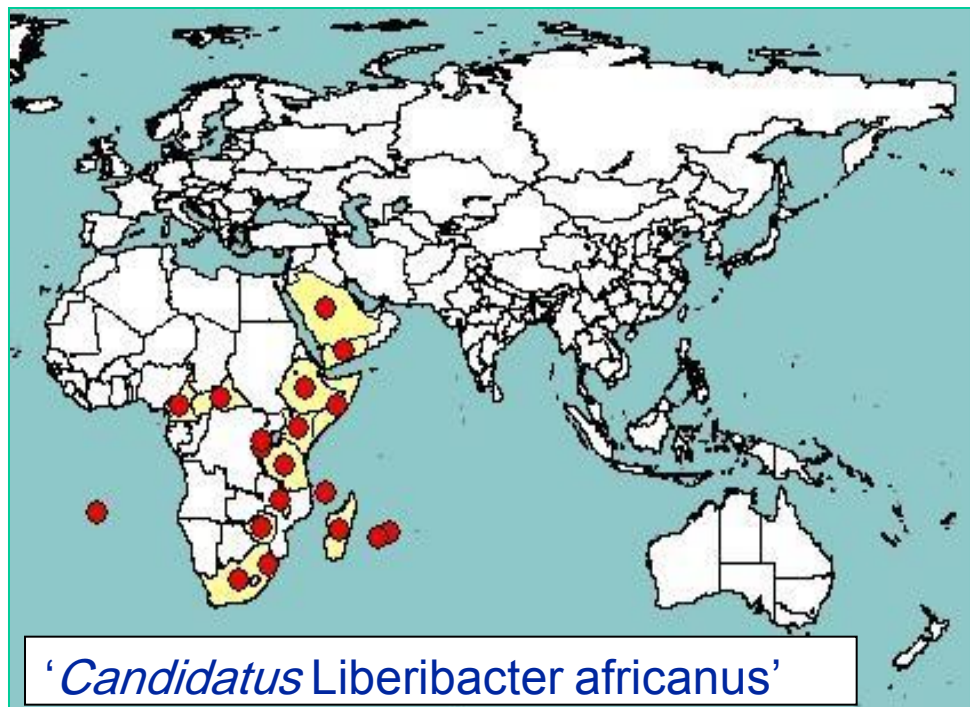
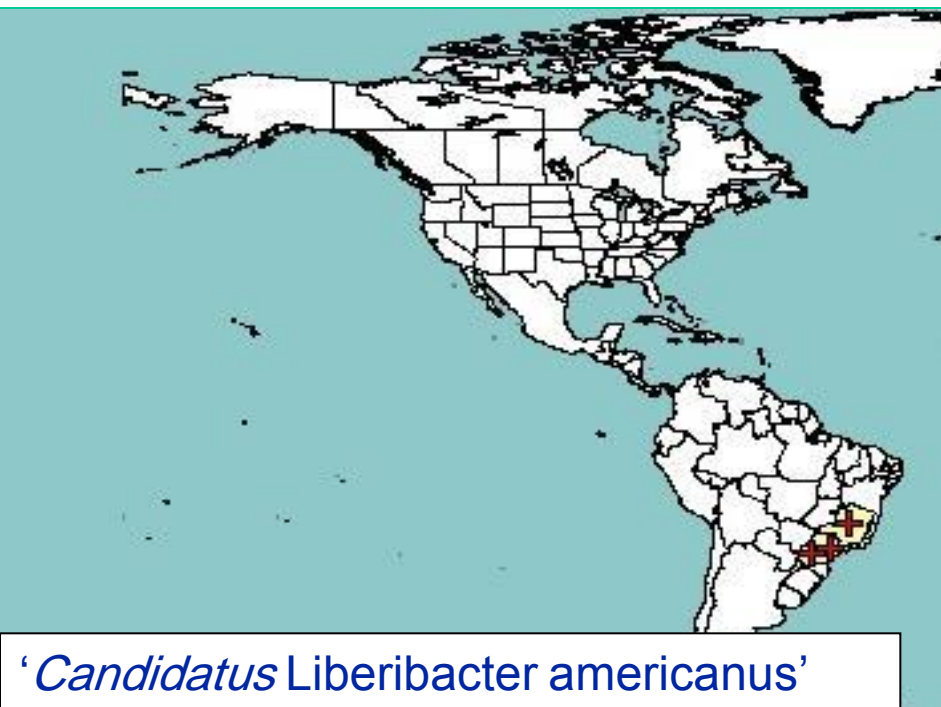
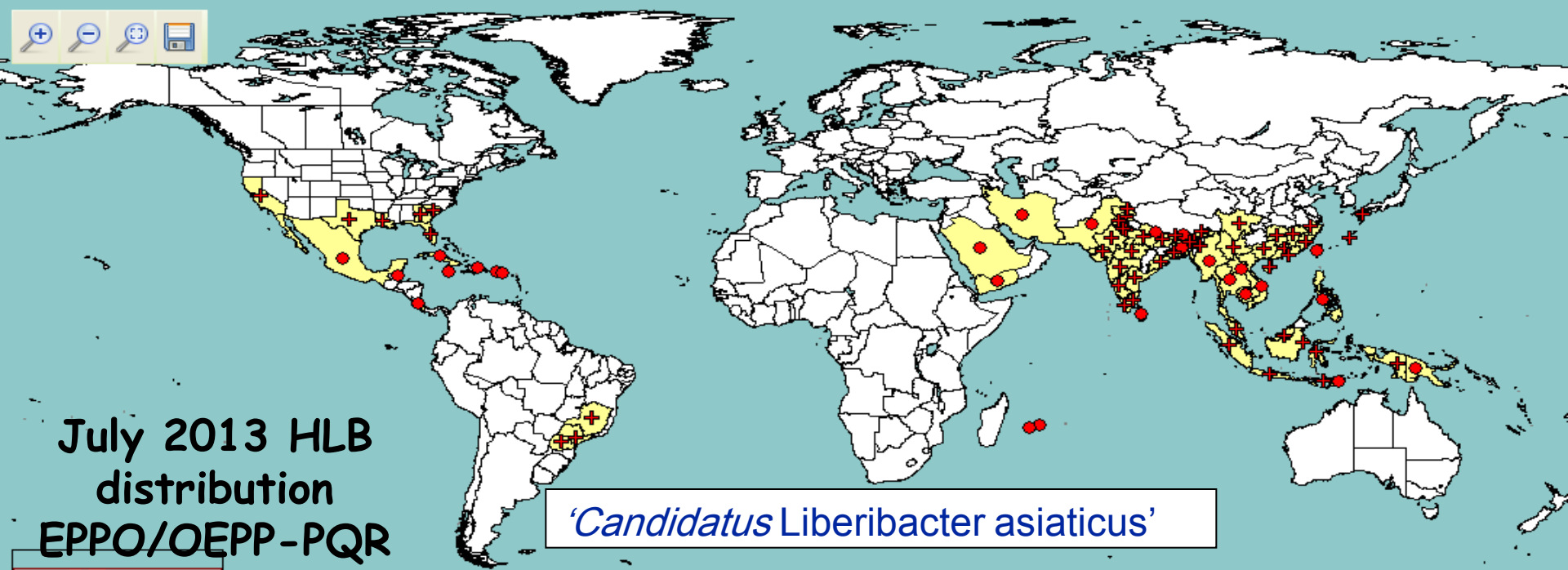


CAUSAL AGENT OF "HUANGLONGBING"



- '*Candidatus Liberibacter asiaticus*'
- '*Candidatus Liberibacter africanus*'
- '*Candidatus Liberibacter americanus*'

- First description of the disease in 1919 in China
- Reported in more than 40 countries
- Phloem-limited bacteria
- Persistently transmitted by several psyllid species
- Some phytoplasmas also involved



HLB AND ITS VECTORS: GEOGRAPHICAL DISTRIBUTION

- Asiatic HLB strains '*Candidatus Liberibacter asiaticus*' (high temperature resistant) and americanus HLB strains '*Ca. Liberibacter americanus*': transmitted by *Diaphorina citri* present in Asia and America



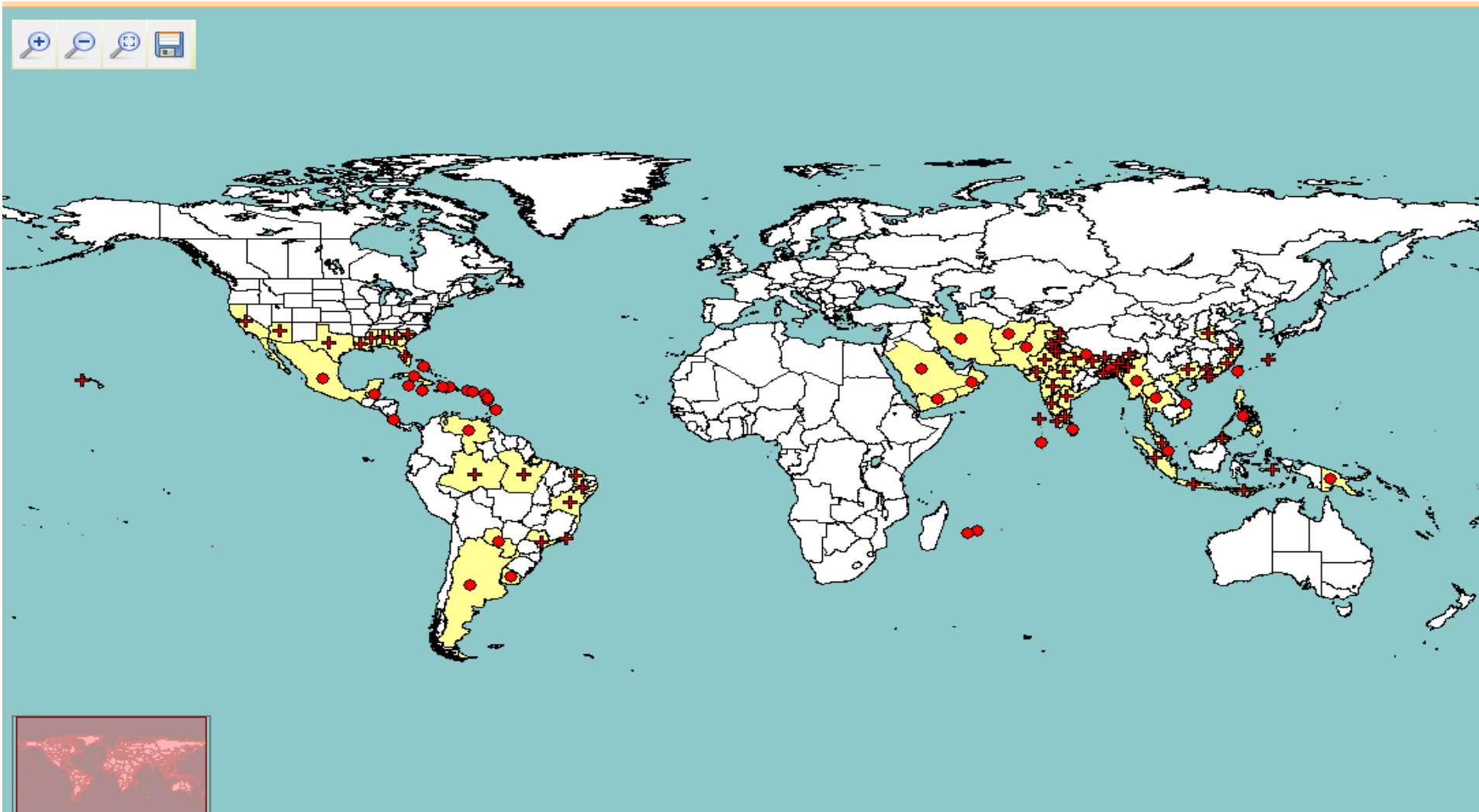
- African HLB strains '*Candidatus Liberibacter africanus*' (sensitive to temperature): transmitted by *Trioza erytreae*, present in Africa (only the vector detected in Canary Islands and Madeira)



- Both vectors are quarantine organisms in the EU

- *Pseudopsylla citrisuga*, recently reported as a new HLB vector in China and *Diaphorina communis* in Buthan.

Diaphorina citri

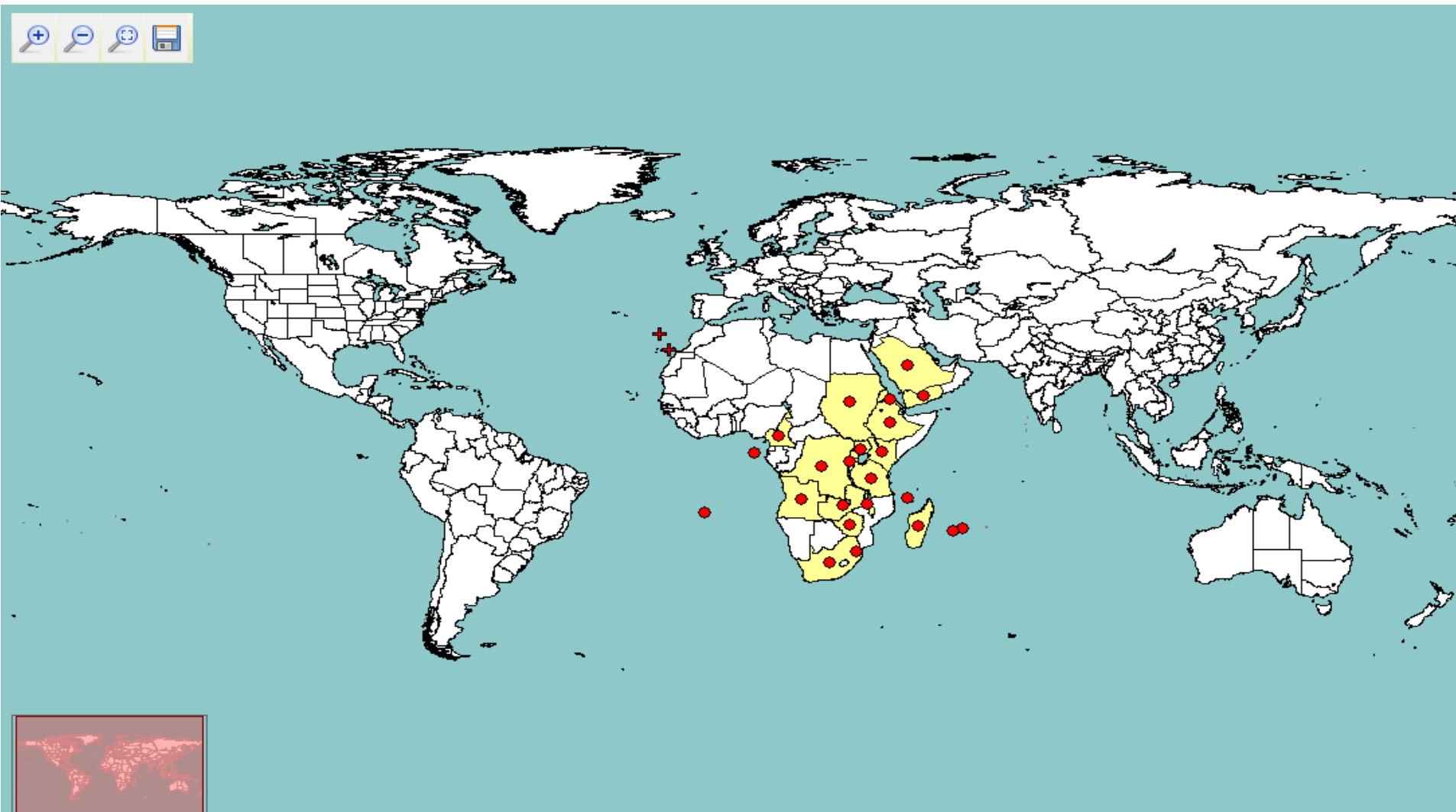


Legend

- Present (national record)
- ✚ Present (subnational record)
- ▲ Transient

July 2013 EPPO/OEPP-PQR

Trioza erytreae



Legend

- Present (national record)
- + Present (subnational record)
- ▲ Transient

July 2013 EPPO/OEPP-PQR

2. Vector transmitting the disease



CYCLE OF HLB

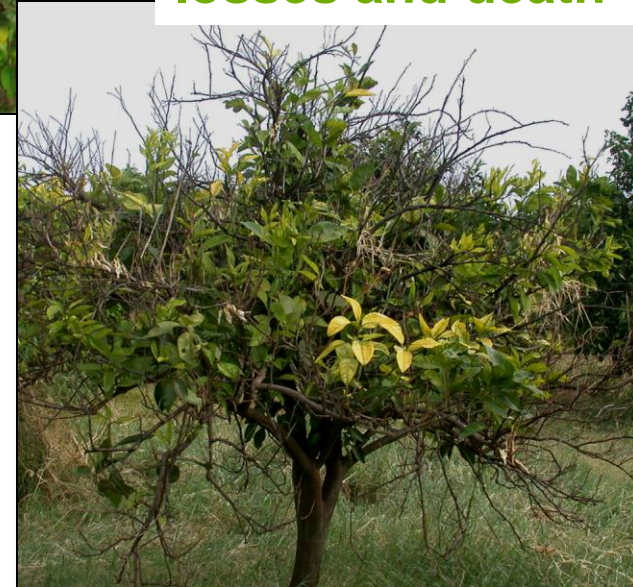
1. Vector feeding in the plant



3. Symptoms appearance



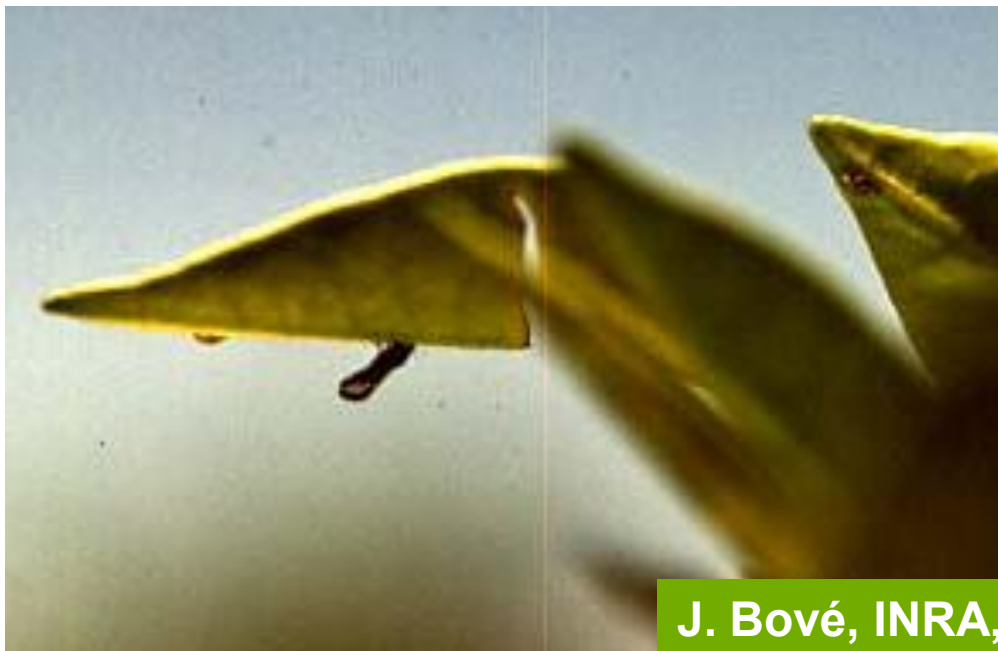
4. Economic losses and death



C. Dardere, SENASA, Argentina

Diaphorina citri (Asiatic psyllid)

	Brazil	USA
<i>D. citri</i>	1942	1998
HLB	2004	2005



J. Bové, INRA, France

Dissemination of HLB in China and in Brazil

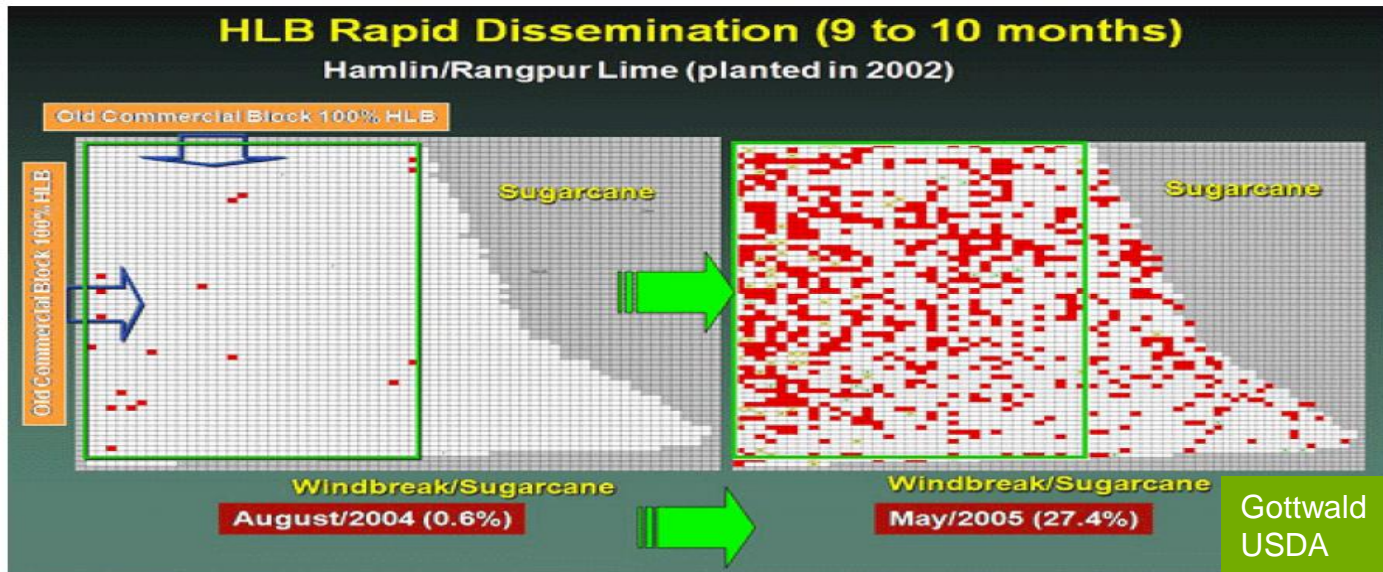
1999



2005



J. Bové, INRA





The Mediterranean region is the fourth citrus producing area in the world and the first exporting fresh fruits

HLB has not been detected yet in the Mediterranean basin

In a near future '*Ca. Liberibacter*' spp. and vectors could entry in the Mediterranean area:

- Preventive measures must be undertaken
- The accurate detection of HLB agents and vectors is essential





Canary Islands (Spain): different stages of *T. erytreae*



Summary of HLB preventive actions in Spain



- Vulgarization of HLB symptoms and risk of introduction of vector species and the bacteria
- Development of an accurate method and a kit for large scale analysis of '*Ca. Liberibacter*' species
- Intensive surveys and analysis of plants and psyllids from the Canary Islands where *T. erytreae* is already present
- Identification of the most threatened citrus areas for the entry of HLB and/or vectors in mainland Spain and preventive surveys
- Sentinel phytosanitary plan for citrus in the Valencian Community (main Spanish citrus producing area): systematic inspections and psyllid species traps followed by lab analyses

HLB preventive actions

International cooperation (since 2006):

INRA-Bordeaux y Univ. V. Segálen Bordeaux 2. France. J. M. Bové.
 FUNDECITRUS. Araraquara, São Paulo, Brazil. J. Ayres, S. Lopes *et al.*
 AGRONOMICA. Porto Alegre, Brazil. P. Telo
 UNIV. ESTADUAL MARINGÁ. Maringá, Brazil. W. Nunes, A.V. Sauer

•LOPES, S.A., et al., (2009). Liberibacters associated with Citrus Huanglongbing in Brazil: 'Candidatus Liberibacter asiaticus' is heat tolerant, 'Candidatus L. americanus' is heat sensitive. *Plant Disease* 93 (3): 257-262.

•LOPES, S.A., et al., (2009). Graft transmission efficiencies and multiplication of 'Candidatus Liberibacter americanus' and 'Ca. L. asiaticus' in citrus plants. *Phytopathology* 99 (3): 301-306.

•BERTOLINI, E., et al. September 2010. Direct procedures for specific detection of 'Candidatus Liberibacter' spp. using immobilized targets and real-time PCR and detection kit. *Spanish patent* 201001157.

•BERTOLINI, E., et al. (2013). Tissue-print and squash real-time PCR for direct detection of 'Candidatus Liberibacter' species in citrus plants and psyllid vectors (Submitted).

Extension publications:

EMERGENCIAS 1422
 Emergencias (Ciencias) 1422

314. Bové y M.M. López / N. Durán-Vila

EVITAR EL HUANGLONGBING (HLB), UN RETO PARA LA SUPERVIVENCIA DE LA CITRICULTURA ESPAÑOLA

El huanglongbing (HLB) es una enfermedad que en los últimos años viene sufriendo un avance espectacular en España. Aunque sus síntomas, asociados a la defoliación y a la caída de la fruta, son similares a los de otras enfermedades de los cítricos, su etiología es diferente. En este artículo se describe el estado actual de la enfermedad en España y se analizan las medidas que se están tomando para evitar su propagación.

En el número 385 de esta revista publicado en 2006 alertamos acerca de la necesidad de evitar la introducción y posterior dispersión del HUANGLONGBING (enfermedad también conocida como "amarillo") y que hoy en día es la enfermedad más devastadora de los cítricos. Las primeras observaciones acerca de los síntomas de esta enfermedad datan de finales del siglo XIX en China pero actualmente se halla muy extendida en varias zonas cítricas de Asia, África y América. Su emergencia, efecto sobre el cultivo y rápida dispersión en el continente americano, llevan incluso a cuestionarse si será posible la supervivencia de la citricultura en algunas zonas de los países afectados.

Síntomas

El síndrome más característico del huanglongbing (que de aquí en adelante designaremos como HLB) y que significa "enfermedad del brote amarillo" en el dialecto local del lugar de China donde se describió por primera vez, se refiere al sistema observado en los primeros estados, en los que los árboles afectados muestran un amarilleo inespecífico de los brotes jóvenes (Fig. 1A).

En marajo defice, este amarilleo inicial progresa afectando a un sector de la copa (Fig. 1B) y eventualmente toda ella. Las ramas afectadas pueden presentar defoliación, muerte de ramillas (Fig. 1C), hojas con un moteado difuso que a veces se encuentra acompañado de síntomas de deficiencias nutricionales, y caída de frutos. En Brasil, Florida y Cuba, como el cítrico permite varias brotaciones, las plantaciones jóvenes se encuentran más afectadas que las plantaciones viejas en las que las brotaciones son menos abundantes.

ESTADO DE ALERTA!
 EL HUANGLONGBING ES LA ENFERMEDAD MÁS DEVASTADORA DE LOS CÍTRICOS

N. Durán-Vila, M.M. López, J.M. Bové

IVIA. Centro de Protección Vegetal y Biotecnología, Moncada (Valencia).
 *Universitat Vicar Segálen Bordeaux 2, Bordeaux, Francia.

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[Cítricos]

EVITAR EL HUANGLONGBING

Una buena defensa es el mejor ataque contra la enfermedad

M.C. Vives
 M.M. López
 L. Navarro

Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias

El huanglongbing (HLB) es actualmente la enfermedad más devastadora que afecta a los cítricos. Esta enfermedad causa graves pérdidas económicas en todos los lugares donde está presente ya que es muy destructiva y difícil de controlar. Está causada por una bacteria que se localiza en el floema de las plantas y que provoca inicialmente el amarilleo de brotes, afecta a la calidad de la fruta y acaba con la muerte del árbol.

Los países citricos de la Unión Europea están en alerta ante la llegada del HLB. No existe ninguna variedad comercial de cítricos ni ningún patógeno que sea resistente a esta enfermedad, por lo que es imprescindible estar que el HLB llegue a las regiones citricolas de España, ya que consera datos colectados en otros países, han saltado debido a un reciente emergencia en el continente americano, donde está devastando la citricultura de los grandes potencias como son Brasil y Florida (Estados Unidos) (Bové, 2006).

Huéspedes y síntomas

Todos las variedades comerciales de cítricos son susceptibles a HLB independientemente del patógeno usado. Los síntomas más severos se han observado en naranjo dulce, mandarina, toronja y pomelo. En los limones y naranjos amargos los síntomas son menos graves y las líneas de resistencia son más abundantes. En los primeros estados de la infección los árboles afectados presentan uno o varios brotes amarillos

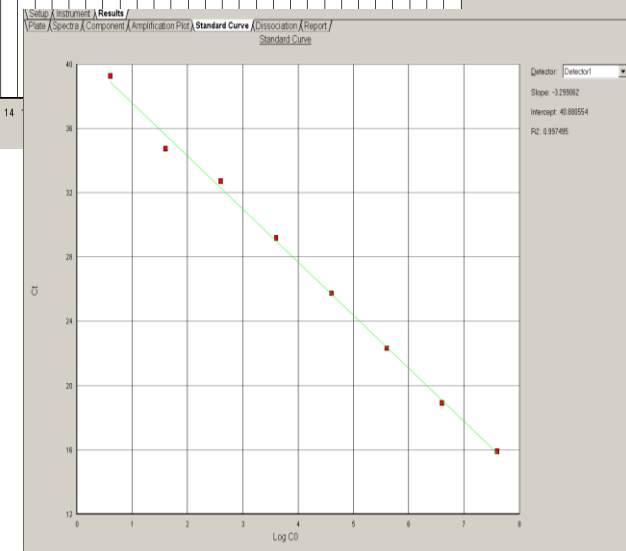
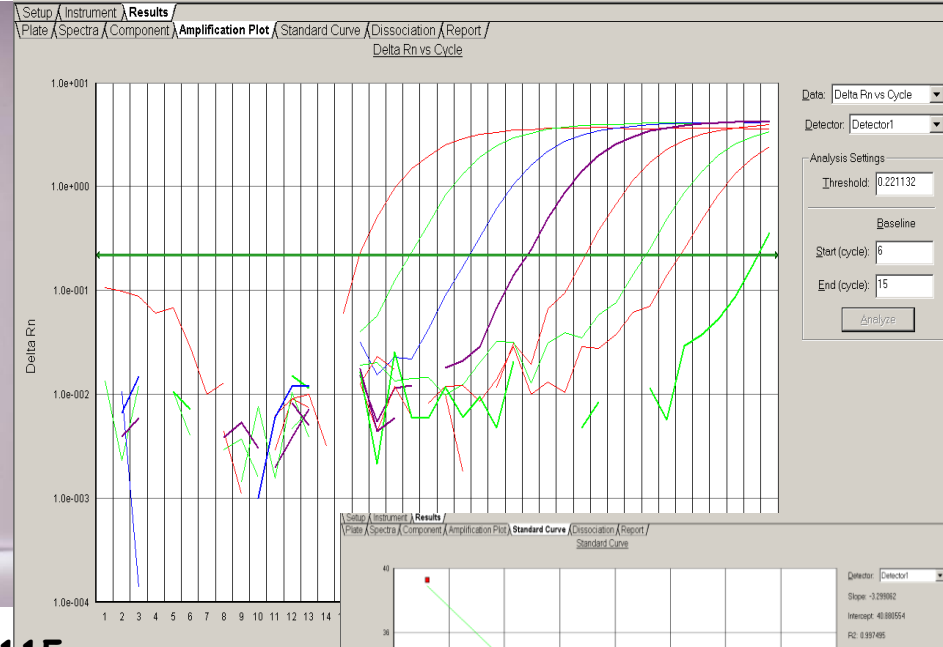


Fig. 1. Aparición general de síntomas de infección directa afectada por HLB. A) Brotes amarillos en una rama. B) Proceso de muerte amarillos en una rama de la copa del árbol.

Conferences:

- 2007: Moncada
- 2008: Huelva
- 2009: Murcia
- 2010: Tenerife
- 2012: Tenerife and La Palma
- 2013: Valencia

Real-time PCR is a very appropriate technique for sensitive detection, but the need for nucleic acids purification greatly limits the number of samples than can be processed and increases the time as well as the cost of the analyses.



Li *et al.* (2006), *J. Microbiol. Methods* 66, 104-115

Teixeira *et al.* (2008), *Mol. Cell. Probes* 22, 139-150

Manjunat *et al.* (2008), *Phytopathology* 98, 387-396

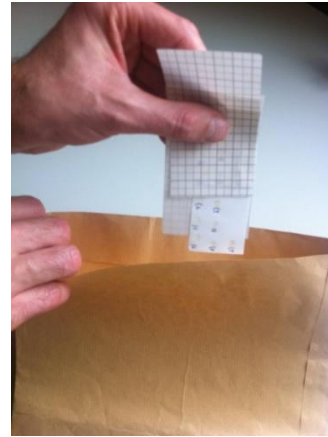
Lopes *et al.* (2009), *Phytopathology* 99, 301-306

Coletta-Filho *et al.* (2010), *Eur. J. Plant Pathol.* 126, 53-60

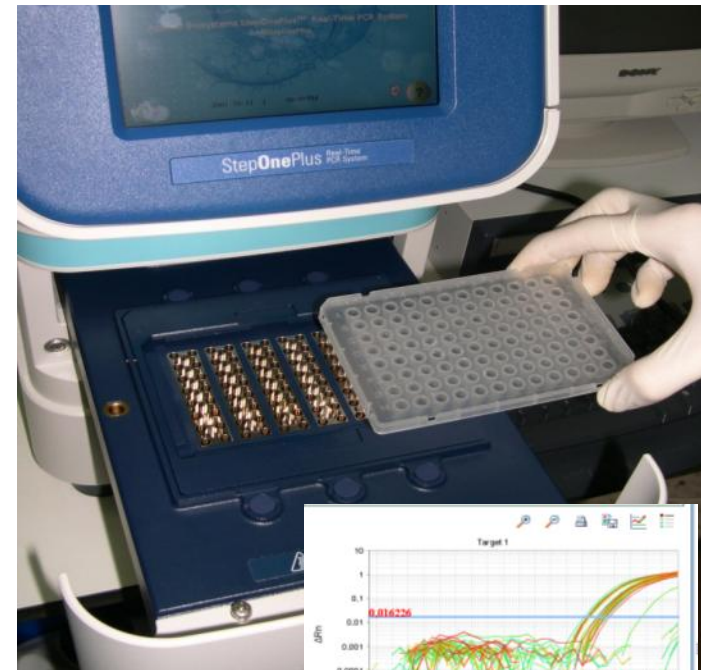
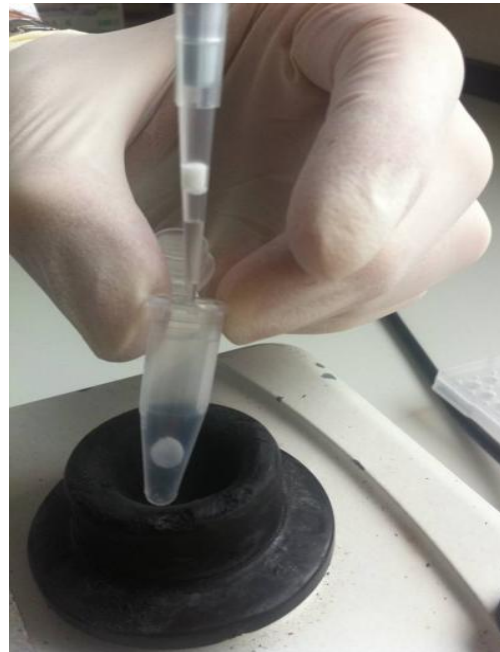
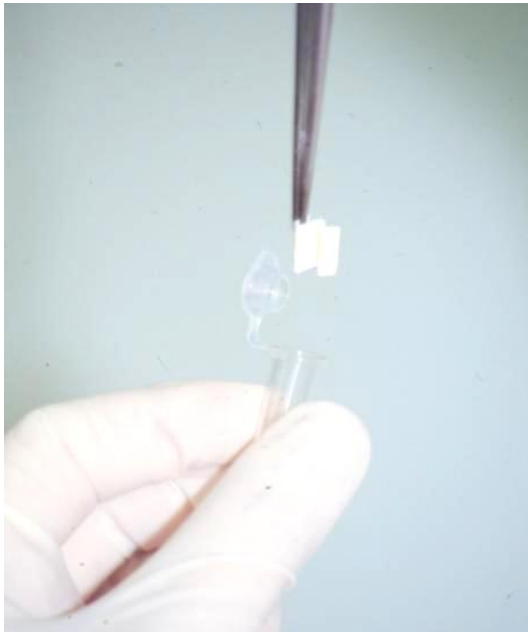
Morgan *et al.* (2012), *Mol. Cell. Probes* 26, 90-98

The main goal was to develop and validate a fast and simple screening test for accurate detection of '*Ca. Liberibacter*' spp. by real-time PCR using direct methods of sample preparation (plant tissues and individual psyllids)





Tissue print real-time PCR



Tissue print real-time PCR

1. **Sample preparation: immobilization of samples by tissue print** of leaf pedicels and/or plant extracts and/or squash of insects on paper membrane. Storage: room temperature.
2. **Extraction of immobilized targets from the membrane** by DN/Rnases free distilled water and vortex.
3. **Preparation of the reagents** and controls supplied in the kit.
4. **Real-time PCR amplification** and interpretation of results.

Rapid screening test based on real-time PCR and direct methods: Performance study

- 30 laboratories from 15 countries were involved.
- 10 blind samples (3 Las, 2 Laf, 1Lam and 4 healthy) immobilized on paper.
- A complete kit Ref. HLB/100 (Plant Print Diagnostics) was sent by courier at room temperature in November 2012.
- Total 400 data that were used for the estimation of the diagnostic parameters, in regardless of the specific involved laboratory.

www.plantprint.net

PlantPrint

Diagnòstics, S.L.

**COMPLETE KIT® FOR "Candidatus Liberibacter" spp.
ASSOCIATED TO CITRUS HLB DISEASE.
RAPID SCREENING TEST BY REAL-TIME PCR**

Kit CaLsp/100 components:

- 1) 2 vials (white cap) containing 50 pieces (0.2 cm²/piece) of Whatman 3MM paper.
- 2) 1 package containing 2 paper membranes (7x13cm) (Whatman 3MM).
- 3) 1 vial (blue cap) containing 1mL distilled sterile water DN/RNases free for master mix preparation.
- 4) 1 tube x 12 mL of distilled water DN/RNases free for sample preparation.
- 5) 2 vials (yellow cap) containing lyophilized complete master

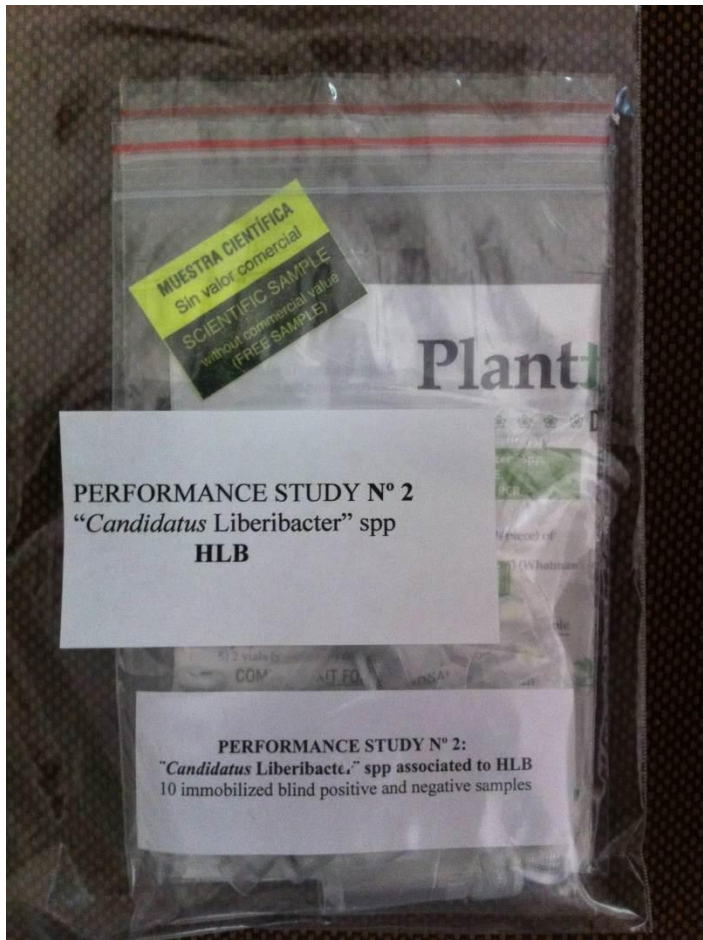
mix (universal primers and TaqMan FAM/TAMRA probe) for "Ca. Liberibacter" spp. amplification (each tube contains enough master mix for 50 reactions using 9 µL/reaction).

- 6) 2 vials (red cap) containing 1 immobilized positive control spotted on a piece of membrane (5 µL per spot of "Ca. Liberibacter asiaticus" infected plant crude extract).
- 7) 2 vials (green cap) containing 1 immobilized negative control spotted on a piece of membrane (5 µL per spot of healthy plant crude extract).
- 8) 1 detailed protocol

© Plant Print Diagnostics, S.L. Complete kit. Spanish-EU Patent N° 2.377.690 (201001157/08-09-2010). IVIA-FUNDECITRUS.

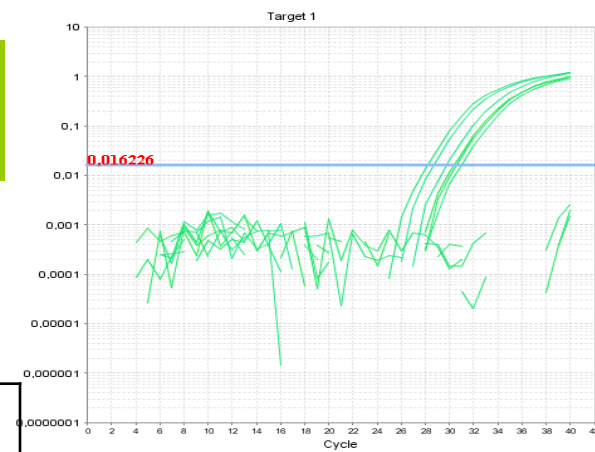
E. Bertolini, M. Cambra, P. Serra, M.M. López, N. Duran-Vila, J. Ayres and J.M. Bové. Direct procedure for specific detection of "Ca. Liberibacter" spp. by immobilized targets and real-time PCR and kit for its detection.





Performance study results

	HLB	NO HLB
POSITIVE	233 (True Positives)	10 (False negatives)
NEGATIVE	7 (False positives)	150 (True negatives)



Total analyses: 400

SENSITIVITY: $0,97 \pm 0,008$

True positives/(True positives + false positives)


SPECIFICITY: $0,94 \pm 0,012$

True negatives/(True negatives + false negatives)

ACCURACY: **96.00 %**

(True positives + true negatives/Total analyses)

Squash real-time PCR for "*Ca. Liberibacter*" spp. detection in psyllids

	Vector	Squash real-time PCR		
		' <i>Ca. Liberibacter americanus</i> '	' <i>Ca. Liberibacter asiaticus</i> '	' <i>Ca. Liberibacter africanus</i> '
<i>Diaphorina citri</i> (Brazil)		5/201	471/1,158	NT
<i>Trioza erytreae</i> (Canary Islands)		0/686	0/686	0/686



Due to the advantages of this screening test it was included in:

COUNTRY CONSULTATION deadline 2013-06-12

113-18639 (12- 18211, 12- 18199, 12- 18144 rev, 11-17247)

PM 7/

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

Diagnostics

Diagnostic

'Candidatus Liberibacter africanus'

'Candidatus Liberibacter americanus'

'Candidatus Liberibacter asiaticus'

Specific scope

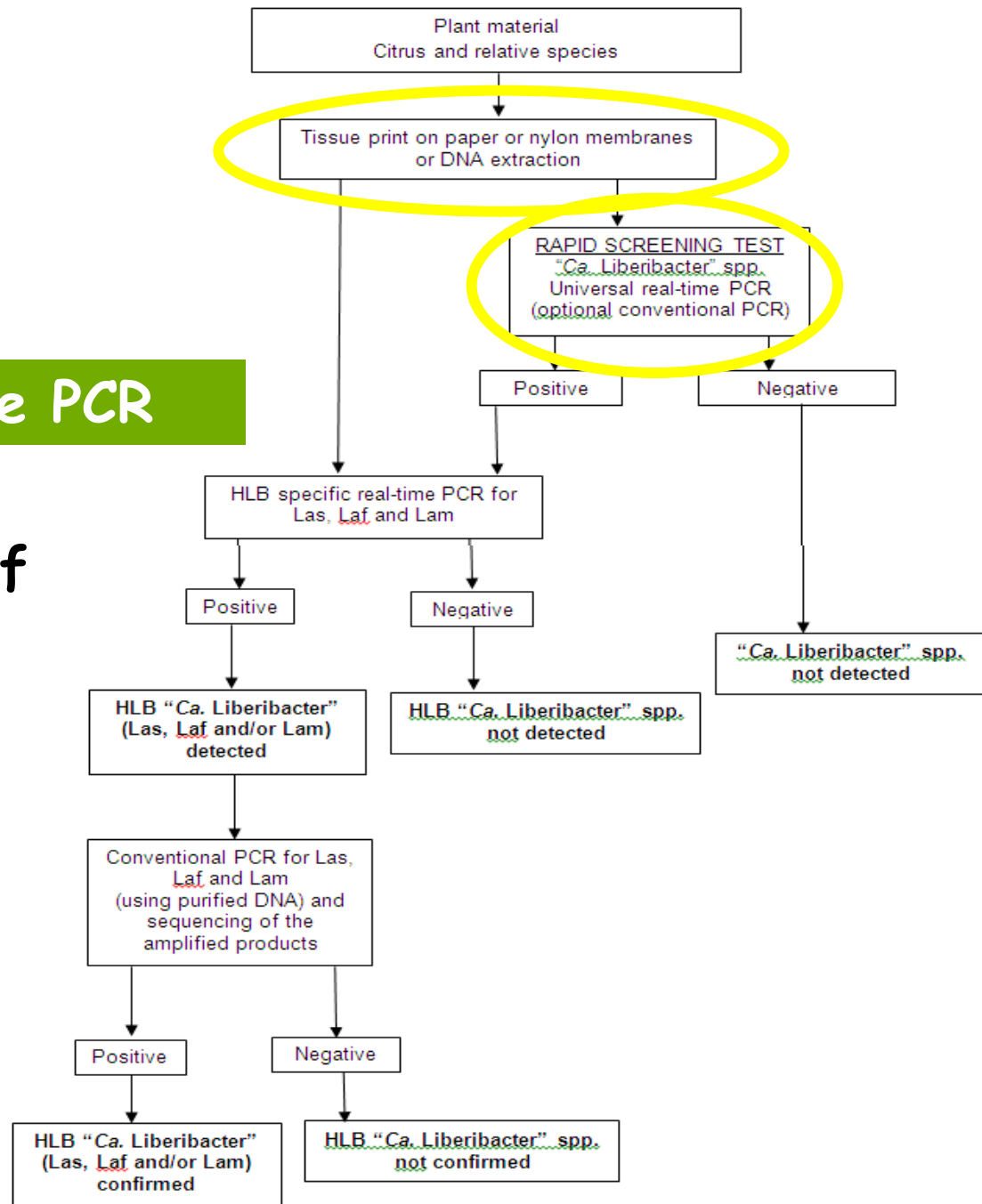
This standard describes a diagnostic protocol for *'Candidatus Liberibacter africanus'*, *'Candidatus Liberibacter americanus'* and *'Candidatus Liberibacter asiaticus'*¹ and for their detection in their psyllid vectors *Diaphorina citri* and *Trioza erytreae*.

Specific approval and amendment

Introduction

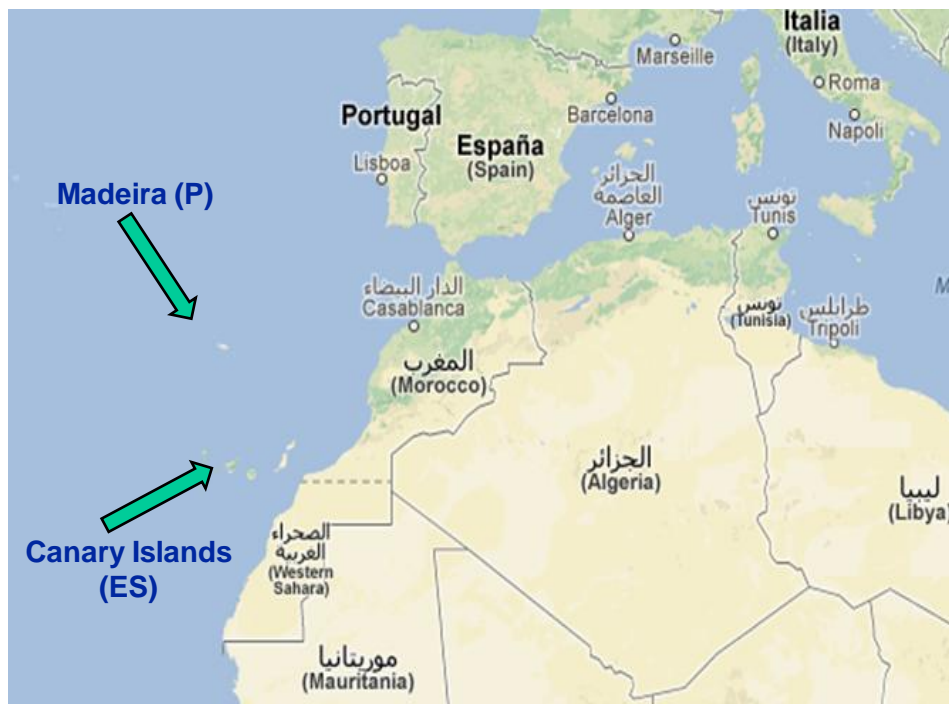
Huanglongbing also called greening, or yellow-shoot-disease, was first described in Southern China in the 19th century, and has been present for many years in the main citrus growing regions of Asia and Africa, being considered the most destructive bacterial disease of the citrus industry (Bové 2006). Huanglongbing is present in more than fifty countries in Asia, Africa, Oceania, and America. Only the citrus producing countries of Mediterranean basin are still free. Practically all commercial citrus species and cultivars are susceptible, regardless of rootstocks (Bové, 2006; Lopes *et al.*, 2009). The pathogen has also been detected in orange jasmine (*Murraya paniculata*). The disease has been associated with three species of bacteria recognized as *'Candidatus Liberibacter'*

Fig. 1: Flow diagram for the detection of "*Ca. Liberibacter*" spp. in plant material.



Tissue print real-time PCR

Proposal of a flow diagram for detection of '*Ca. Liberibacter*' spp.



Canary Islands surveys: 2009 to 2013

14270 visually inspected trees
(935 sampled and tested) and 783
T. erytreae individually analyzed

**NO DETECTION OF HLB
AGENTS**





PREVENTIVE SENTINEL PLAN FOR CITRUS IN THE VALENCIAN COMMUNITY OF SPAIN

“PLAN DE VIGILANCIA FITOSANITARIA DE CÍTRICOS”



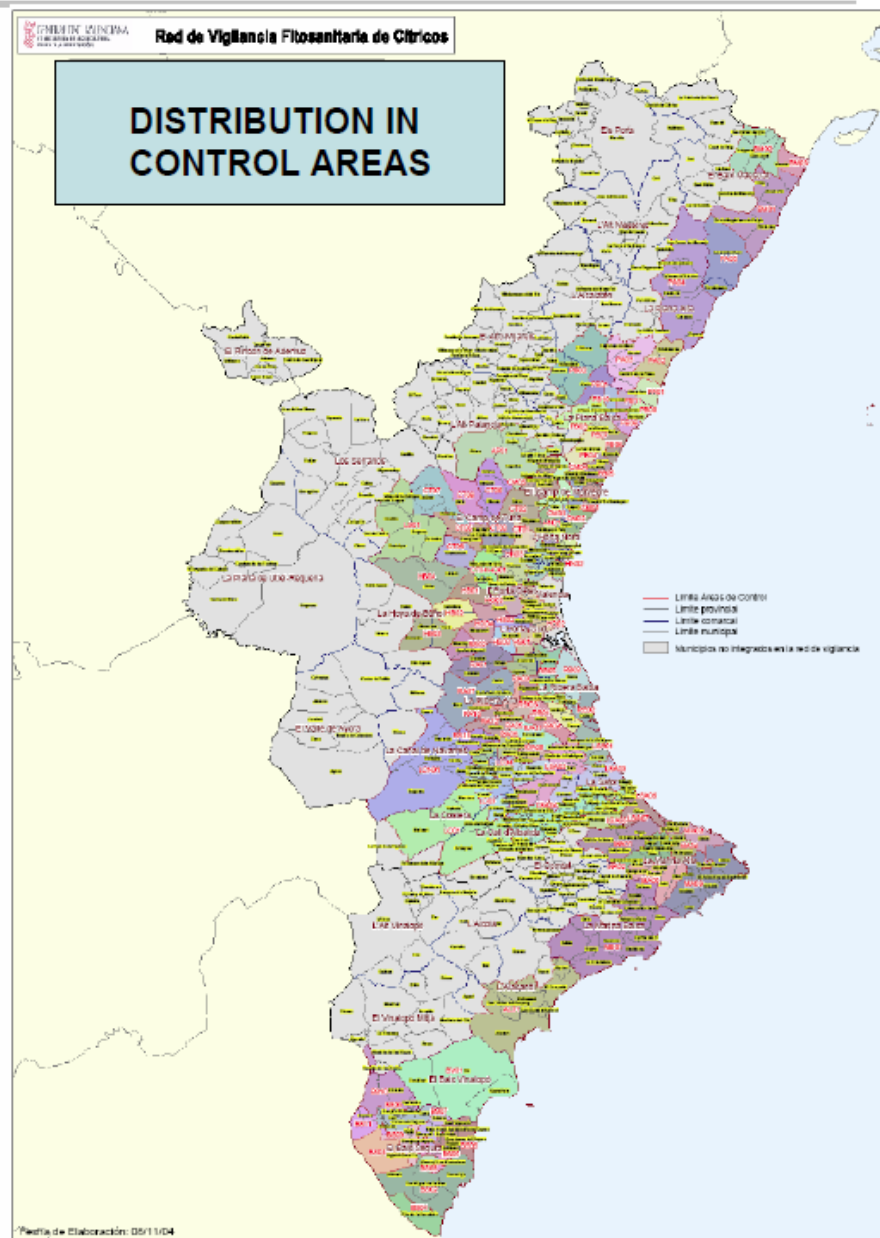
The whole citrus surface of Valencia (180,000 ha) was divided in 100 control fixed areas (selected orchards):

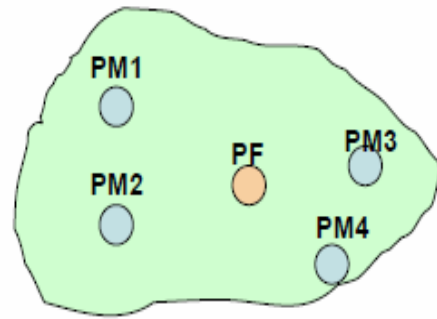
- 20 in Castellón
- 60 in Valencia
- 20 in Alicante

In addition 17 strategic points were selected:

- 2 international airports
- 3 ports
- 4 farms
- 8 packinghouses

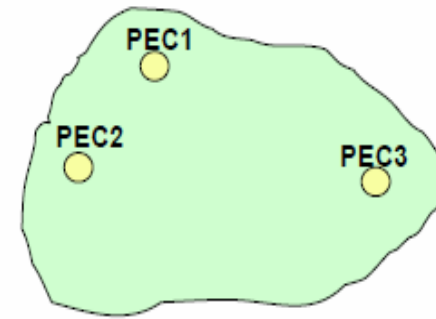
Traps were placed in all the fixed plots and strategic points





4 aleatory sampling
(PM) & 1 fixed (PF)

+



Additionally all the
Estrategic points (PEC)

+

Two main tasks:

- 1) Visual inspection for quarantine and common pests and diseases in PM & PFs.
- 2) Traps for quarantine pests in PF & PEC.

3,000 trees/year/inspector (carefully inspected in areas with traps)



Strategic points:

- Airports: Alicante & Valencia.



- Ports: Gandía, Valencia & Castellón.



- Farms and livestocks where citrus are used: 4



- Warehouses where citrus imported from third countries are processed (sorting, re.packing, etc.): 8



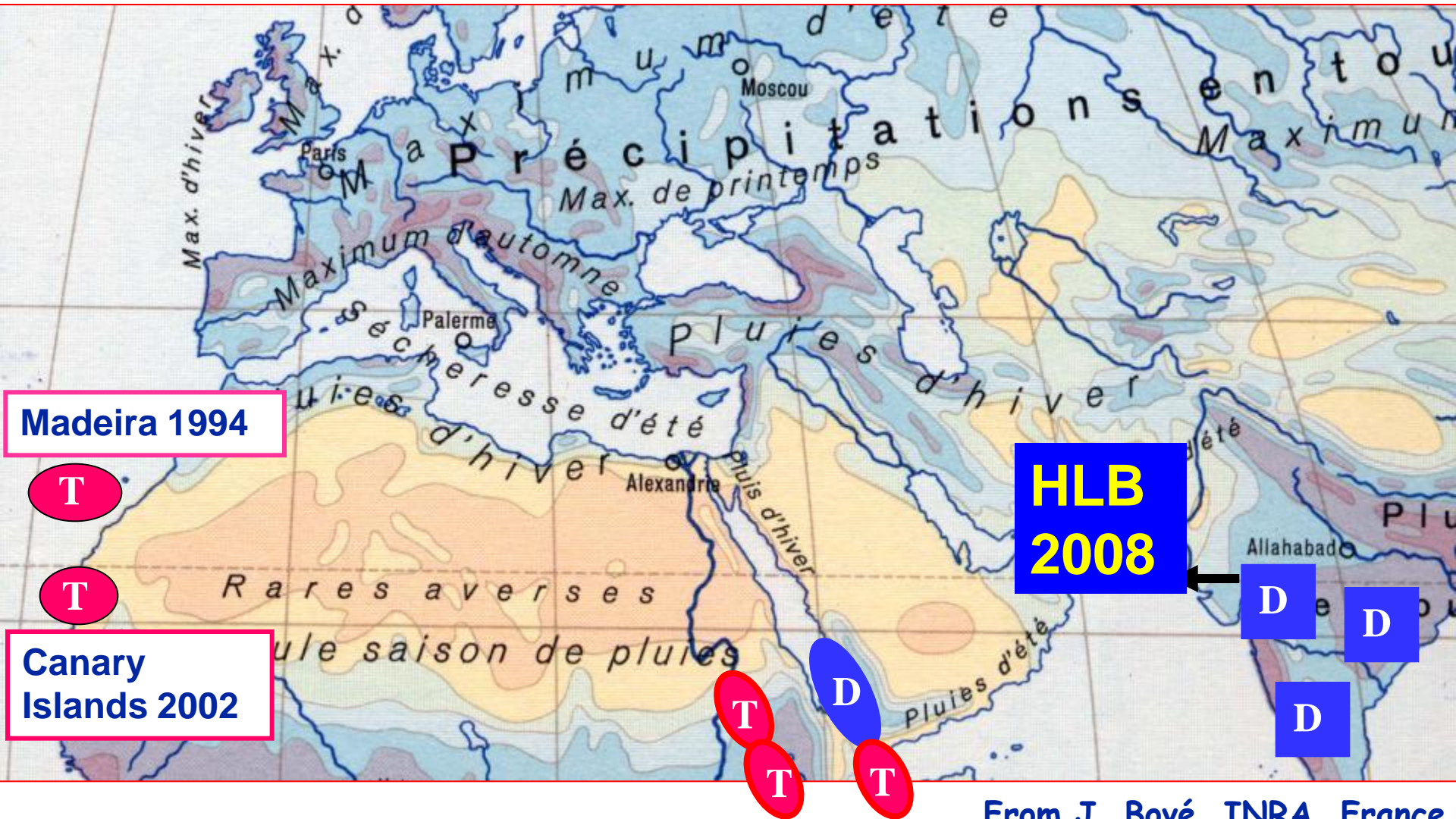
Results of the sentinel plan

- It has allowed the detection of several new pests and diseases
- None of the identified psyllid species were among the reported HLB vectors
- 62,500 inspected trees: 1,385 analyzed samples. No '*Candidatus Liberibacter*' species causing HLB were detected
- This type of surveys should be implemented in all Mediterranean citrus producing countries

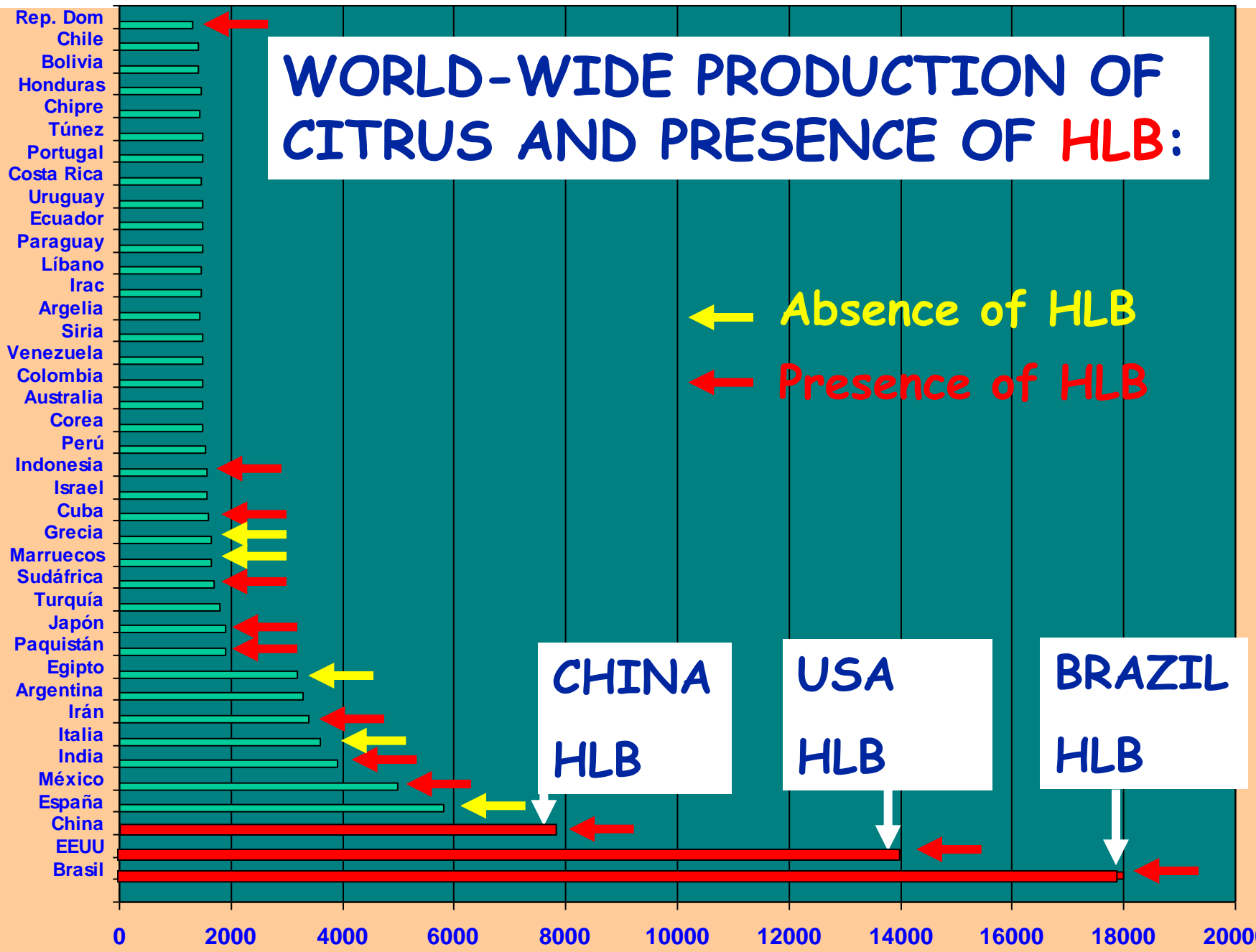
MEDITERRANEAN REGION: STILL HLB FREE BUT

● : *Trioza erytreae* ●D : *Diaphorina citri*

...ARE APPROACHING



WORLD-WIDE PRODUCTION OF CITRUS AND PRESENCE OF HLB:



CHINA
HLB

USA
HLB

BRAZIL
HLB

Conclusions

- The economic and social importance of Mediterranean citrus industry justifies actions against HLB: prevention is essential
- In Spain, practically 100% of citrus orchards are based on certified plant material locally produced. This is a guarantee of their sanitary status and is currently a big advantage
- The availability of rapid, simple and efficient diagnostic methods is essential for surveys and for rapid eradication, if necessary
- The Mediterranean citrus industry should be able to avoid the introduction of infected material from third countries and of HLB vectors to maintain its economic importance in the next future

THANK YOU VERY MUCH FOR YOUR
ATTENTION
and Prof. J. Bové for information

