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RESEARCH PAPERS

Survey of huanglongbing associated with '*Candidatus* Liberibacter' species in Spain: analyses of citrus plants and *Trioza erytreae*

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Summary. The disease huanglongbing (HLB), caused by the phloem-limited and psyllid-vectored '*Candidatus* Liberibacter' spp., is threatening the Mediterranean citrus industry. The African psyllid (*Trioza erytreae*) vector of the pathogen was detected in Madeira (Portugal) in 1994 and in the Canary Islands (Spain) in 2002, and its arrival in 2014 in northwest Spain and Portugal along the Atlantic coast instigated a biological alert, and a contingency management plan was developed. Extensive surveys were conducted in Canary Islands from 2009 to 2015 and in the northwest mainland Spain (Galicia) since the first detection of *T. erytreae*. Symptoms of the psyllid were observed in most sweet orange orchards of five islands in Canary Islands (93% of the inspected plots). In northwest mainland Spain, 65% of the inspected plots up to 2016 showed *T. erytreae* symptoms. During the surveys, ten leaves/tree from trees showing suspicious symptoms and from symptomless trees, as well as adult psyllids, were collected and analysed by real-time PCR using a universal '*Ca*. Liberibacter' spp. kit, according to the EPPO standard. Suspected samples from other surveyed Spanish regions free of the vector were also analysed. The few samples that were positive in the screening test were tested by species-specific real-time PCR protocols, and they did not show amplification. These data confirm that the Spanish citrus industry is currently free of the '*Ca*. Liberibacter' spp., but strict measures to prevent the introduction of this pathogen are required as the presence of *T. erytreae* increases the risk of its dissemination.

Key words: Greening, African citrus psyllid, direct methods of sample preparation.

Introduction

The Euro-Mediterranean citrus industry is socioeconomically very important, covering about 12% of the world citrus growing area and producing approx. 20% of the world citrus fruit (faostat.fao.org). Approximately 70% of the citrus fruit productive area is concentrated in four countries: Spain (27%), Italy (16%), Egypt (15%) and Turkey (10%) (Schimmenti et al., 2013); but citrus fruit are also produced in Morocco, Greece, Syria, Israel, Algeria, Tunisia, Jordan and Cyprus, in decreasing order of production volume. Spanish citrus crops cover 320,000 ha with a total production of over 6.5 million tonnes, being the sixth producing country in the world and greatest exporter of fresh citrus fruit (faostat.fao.org). This situation has been possible due to a programme of propagation of selected pathogen-free cultivars

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grafted on Tristeza-tolerant rootstocks, in the framework of a certification scheme (Cambra *et al.*, 2000).

In the Canary Islands archipelago, citrus are grown on five of the seven islands, totalling 1,022 ha of sweet orange and 296 ha of other citrus species. These trees are generally cultivated in small and widely scattered plots, and isolated trees are found in most private and public gardens, approaching over 200,000 trees (SECAGPA, 2012).

In Galicia (northwest quadrant of the Iberian Peninsula) there are no commercial citrus orchards, but approx. 119,500 plants, mainly lemon trees, are located in private and public gardens, representing 221 ha of lemons and 94 ha of sweet orange, mainly in Pontevedra and A Coruña provinces (MAGRAMA, 2015).

Huanglongbing (HLB) or citrus greening disease endangers the citrus industry worldwide (Bové, 2006). This disease has been described in most of the major citrus producing areas of the world, mainly in America (mostly in Brazil, United States of America, and Mexico), China, India and South Africa. Only the Mediterranean basin, Asian subtropical regions, Australia, New Zealand, and North and South Pacific islands are still HLB free (EPPO, 2016a). However, the disease is currently present in the Arabian Peninsula, including Iran and Yemen, and in equatorial and western Africa in areas relatively close to the Mediterranean basin. HLB continues to spread outwards from the Middle East and southern Africa to the North and East territories, representing a serious threat for citrus growing regions that are still HLB-free (Bové *et al.*, 2006; Duran-Vila *et al.*, 2009; 2014; 2015; EPPO, 2016a; Duran-Vila and Bové, 2015; López *et al.*, 2015; Bové and Duran-Vila, 2016). Neither large nor systematic surveys have been organised in the Euro-Mediterranean countries for monitoring HLB and the insect vector.

Typical HLB symptoms in citrus are rapid tree decline, including yellow shoots with blotchy mottled leaves, lopsided fruits with colour inversion, aborted seeds, leaf and fruit drop and shoot dieback (Da Graça 1991, Bové 2006, Wang and Trivedi, 2013). HLB is associated with three species of Gram negative bacteria belonging to '*Candidatus* Liberibacter' and dependent on the temperature for optimal multiplication and titre (Lopes *et al.*, 2009): '*Ca.* L. africanus' (CaLaf) is the heat sensitive species found in Africa, Madagascar, Yemen and Iran; '*Ca.* L. asiaticus' (CaLas) is the heat tolerant species found in Asia, South, Central and North America; and '*Ca.* L. americanus' (CaLam) is a new heat sensitive species only detected in Brazil (Teixeira *et al.*, 2005). These bacteria are restricted to host sieve tube elements, and can multiply in vector insects, apparently as endosymbionts (Subandiyah *et al.*, 2000). Transmission of the bacteria occurs by grafting or vegetative propagation of infected plant material, and naturally by two psyllid species (Hemiptera, *Physillidae*), the Asian citrus psyllid *Diaphorina citri* (Capoor *et al.*, 1967; Yamamoto *et al.*, 2006) and the African citrus psyllid *Trioza erytreae* (McClean and Oberholzer, 1965). Both CaLas and CaLaf can be propagated, respectively, through populations of *D. citri* and *T. erytreae*, by transovarial transmission, and horizontally by sexual transmission from males to females in *D. citri*, although at low rates (Grafton-Cardwell *et al.*, 2013). While *D. citri* is by far the most prevalent HLB vector worldwide, *Trioza erytreae* transmits CaLaf in Africa, parts of Arabia, and in some Indian Ocean and Atlantic Ocean islands (Gottwald, 2010). Recently, *D. citri* has been detected in mainland Africa (Shimwela *et al.*, 2016), although neither CaLaf nor CaLas were detected in insect specimens. Experimentally, both African and Asian citrus psyllids can transmit both CaLaf and CaLas associated to HLB (Bové, 2006). In addition, CaLas has been detected in *Cacopsylla citrisuga* identified in China and in *Diaphorina communis* in Bhutan (Cen *et al*., 2012; Donovan *et al*., 2012), but the ability of these psyllids to transmit the pathogen has not been demonstrated. Reports indicate that when an infected psyllid vector species has been introduced into a new area, HLB is detected and spreads in a variable period. In many countries, the presence of the vector species is generally followed by the detection of one or several '*Candidatus* Liberibacter' species that were already present, but were not able to spread naturally (Bové, 2006).

There has been unexpected and sequential identification of *T. erytreae* in Europe. The first report in 1994 from the island of Madeira (Portugal) considered the insect of unknown origin, but the introduction of infested citrus by sailors from Africa was probable. Since then, the African citrus psyllid has spread to all the islands of the archipelago (Carvalho and Aguiar, 1997; Fernandes and Aguiar, 2001). The insect was first reported in Spain in the Canary Islands in 2002 (Hernández, 2003). In summer 2014 *T. erytreae* was also identified in the Iberian Peninsula in Pontevedra (Galicia, northwest Spain), and in northern Portugal (Pérez-Otero *et al.*, 2015). This situation resulted in a contingency plan developed by the Galician authority in agriculture (Consellería do Medio Rural e do Mar, Xunta de Galicia) on February 10, 2015, in co-ordination with the National Ministry of Agriculture (resolution July 1, 2015), with the goal of pest containment. This was followed by an official declaration of the pest in Galicia (DOG, 2015).

Because there are no curative treatments for HLB, quarantine measures to avoid entry and establishment of the disease, as well as the early detection of the associated bacteria followed by eradication of the first foci, are the most effective strategies to prevent its introduction into new areas. In early years, different methods were developed for detection of HLB associated bacterial agents, mainly including biological indexing, electron microscopy, serological techniques, and dot-blot DNA hybridization (reviewed by EPPO, 2014). Conventional PCR was then applied for universal and specific detection (Jagoueix *et al.*, 1996, Hocquellet *et al.*, 1999 and Teixeira *et al.*, 2005). Development of real-time PCR solved the main sensitivity, specificity and reliability problems by using universal or species-specific protocols (Li *et al.*, 2006; Teixeira *et al.*, 2008, Coletta-Filho *et al.*, 2010; Morgan *et al.*, 2012; Fujikawa *et al.*, 2013; Bertolini *et al.*, 2014). A flow diagramme for the detection of '*Ca*. Liberibacter' spp. associated with HLB in plant material and insect vectors (EPPO, 2014) recommends real-time PCR as a useful tool. This high-throughput technique has improved specificity, sensitivity and accuracy of pathogen detection, and has reduced risks of cross-contamination (López *et al.*, 2009). Moreover, real-time PCR allows the use of direct sample preparation methods such as spot, tissue-print and/or squash tissue systems from targets deposited on membranes, increasing the number of samples that can be immobilized in field conditions, mailed at room temperature to a laboratory and processed daily in a robust and reliable manner (De Boer and López, 2012; Bertolini *et al.*, 2014).

The detection of *T. erytreae* triggered a phytosanitary emergency in Spain, and measures were implemented that include the monitoring of the pest to assess the presence of the vector and extensive surveys of HLB. The objective of this programme was to contain the pest vector and to prevent HLB and its eventual spread to the Mediterranean citrus industry. Some results of the actions carried out to date are presented here, with the aim to encourage surveys in other Mediterranean countries to prevent HLB in the region.

Materials and methods

Field monitoring of *Trioza erytreae* and HLB surveys

Monitoring and surveys were conducted from 2009 to 2015 in the Canary Islands, and following the first detection of the vector in Galicia, these were also carried out in the provinces of Pontevedra, A Coruña and Lugo, in 2014 and 2015. A tree was considered as affected by the psyllid when inspectors observed typical symptoms on young leaves as well as on older leaves of the same vegetative flush. The percentage of trees with damage and the percentage of damaged branches per tree (visual estimation) were recorded.

The number of samples to be taken in each district was determined according to the total area of citrus grown or the officially estimated number of individual trees. Mainly trees affected by *T. erytreae* were sampled, but randomly selected pest-symptomless trees showing yellow shoots, different types of leaf mottle, other HLB-like symptoms or with no symptoms were also sampled. In Canary Islands, at least 10% of the trees in any regular plantation and 100% in small groves were visually inspected for African citrus psyllid, and samples were collected if suspicious symptoms were observed. Individual trees in public or private gardens were also sampled. In Galicia, monitoring and surveys were initially performed following transects from the first detection of *T. erytreae* in Vilanova de Arousa (Pontevedra). In a second phase, the inspections were mainly focused along the coast from A Guardia (Pontevedra) on the border with Portugal through the coast of A Coruña province to Ribadeo (Lugo province). Mainly orchards in the Canary Islands and individual trees from private and public gardens in Galicia were inspected and sampled. Monitoring and surveys were also performed in the main Mediterranean citrus producing regions in Spain, and plants were analysed when any suspicious symptoms were observed.

Plant sample collection and printing and squashing of psyllid species

Two methods of plant sample collection were used. In one, direct tissue-prints were taken in field conditions of ten mature leaf petioles per tree, randomly collected around the canopies of symptomless trees, a few leaves from trees showing suspicious symptoms were selected. The tissue-prints were performed by detaching each fresh leaf by hand using gloves and pressing the petiole on Whatman 3MM paper (GE Healthcare Europe or Plant Print Diagnostics) or on nylon positively charged (Roche) membranes. The overlapping prints of ten petioles were made on an area of about 0.5 cm^2, according to Bertolini *et al.* (2014) and EPPO (2014). The printed membranes containing immobilized samples were kept at room temperature in dark and dry conditions until analyzed, or mailed to laboratory. For the second method, five shoots cut with scissors around the canopy of each tree or several trees, showing HLB-like symptoms, were collected. The shoots were introduced into a plastic bag, labeled and kept in a refrigerated box. In the laboratory, two mature leaves were detached by hand from each shoot and the petioles were printed on membranes as described above, a few days after sample collection.

Trioza erytreae specimens, identified according to the EPPO (2005) protocol, were collected with the aid of a mouth aspirator from each plant. The insects were directly deposited with small brush onto the surface of a membrane (described above) and individually squashed until total disruption using the round bottom of an Eppendorf tube. Psyllids were squashed (EPPO, 2014; Bertolini *et al.*, 2014), and the membranes were stored at room temperature in dark and dry conditions or mailed to the laboratory. Psyllids were also collected and introduced into tubes containing 70% ethanol and stored in the dark until use. The storage of psyllids preserved in alcohol and squashed membrane was as described above. Occasionally, *T. erytreae* individuals stored in alcohol were mailed to a laboratory for species confirmation or for analysis.

DNA extraction

Alternatively to the previously described direct methods of sample preparation, DNA was purified from mature leaf samples according to methods described in EPPO (2014). Extracts from mature sweet orange or citron leaves (0.5 g of petioles and leaf midribs) were homogenised in individual plastic bags with heavy net (Plant Print Diagnostics) using a hammer, or into plastic bags with thin net (Bioreba) using a Homex 6 homogenizer (Bioreba). In both cases, the plant material was ground at about 1;20 (w;v) in PBS buffer pH 7.2 supplemented with 0.2% (w:v) sodium diethyl dithiocarbamate. Total DNA was extracted using the simplified cetyltrimethyl ammonium bromide (CTAB) method of Murray and Thompson (1980) according to EPPO (2014), or using the DNeasy Plant Mini Kit (Qiagen) following manufacturer's instructions. For *T. erytreae* DNA extraction, the same procedures were used after homogenisation of a single specimen into an Eppendorf tube using the extraction buffer described above. The DNA was stored at -20ºC until use.

'*Ca*. Liberibacter' spp. detection by real-time PCR and specific '*Ca*. Liberibacter' species amplification

The complete CaLspp/100 kit (Plant Print Diagnostics) was used for real-time PCR detection, based on the universal primers and TaqMan probe described by Bertolini *et al.* (2014) as rapid screening tests according to EPPO (2014). Tissue-printed plant samples or squashed psyllids were processed according to manufacturer's instructions. Pieces of membranes with the printed plant samples or squashed psyllids were carefully introduced with tweezers into Eppendorf tubes, 100 µL of distilled water were added and the mixture was vortexed, placed on ice until amplification, and stored at -20ºC after membrane removal. The real-time protocol of the kit used 3 or 5 µL for amplification and an annealing temperature of 64ºC. The protocol was also used with purified DNA as target. Any positive sample obtained in this screening test was tested by the species-specific primers and TaqMan probe described in Li *et al.* (2006) against '*Ca*. L. africanus', '*Ca*. L. americanus' and '*Ca*. L. asiaticus' (EPPO, 2014). The commercially available kits used for the detection tests included Whatman 3MM paper immobilized positive controls of bacterial DNA (chimera of '*Ca*. Liberibacter' spp. associated to HLB-DNA immobilized on paper) diluted 1:100 in sweet orange crude extract prepared in PBS. This DNA is a construction in a cloning vector with the target DNA for the different species of '*Ca*. Liberibacter' (Plant Print Diagnostics). The HLB-ID complete kit (Plant Print Diagnostics), containing positive controls immobilized on paper, was also used for specific amplifications. Positive samples were also tested against '*Ca*. L. solanacearum' using the complete kit CaLsol/100 (Plant Print Diagnostics) that also uses a TaqMan probe based on Teresani *et al.* (2014). The extracts positive by the universal kit, stored at -20ºC after membrane removal, were used for the species-specific amplifications. Amplification reactions were performed in StepOne Plus (Applied Biosystems), Light Cycler 480 (Roche) and/or Smart-Cycler (Cepheid) thermocyclers.

Results

Canary Islands

Trioza erytreae was localized in the islands of Tenerife, La Palma, La Gomera, Hierro and Gran Canaria, but not in Lanzarote, Fuerteventura and La Graciosa, where a few citrus plants are grown mainly as ornamentals (Figure 1). The pest vector was identified along the north coast of Tenerife, and then a total of 210 commercial plots were monitored and surveyed in several islands, representing 199.6 ha of citrus orchards (Table 1). *Trioza erytreae* symptoms were observed in most sweet orange orchards of the Canary Islands (93% of the inspected plots), with severe damage in the northern slopes of the islands which experience humid and warm weather. On the islands where *T. erytreae* has been detected, almost in all the monitored commercial plots (97%), the percentage of trees showing *T. erytreae* damage reached 90%, and 37.1 ± 14.7 % of the shoots exhibited nymphal bumps on the upper leaf surfaces corresponding to nests on the lower leaf surfaces (Figure 2A, B and C). Typical ovoid eggs, some of the five nymphal instars, or winged male and female adults were sometimes observed on the affected trees (Figure 2C, D and E). However, on many occasions, living *T. erytreae* eggs, nymphs or adults were not found in the visited plots, although trees were affected, since symptomatic leaves remain on trees for long periods. The most affected citrus species were usually lemon and mandarin trees, whereas sweet and sour orange trees were comparatively less damaged by the insect.

Table 1 shows the monitoring and survey results achieved in 7 years (2009 to 2015) in Canary Islands in areas where *T. erytreae* was present, and results of the HLB survey. From a total of 108,270 inspected trees in the Canary Islands, 3,355 were analysed as well as 1,464 *T. erytreae* individuals. The only suspicious symptoms observed were yellowing and chlorosis of the leaves; however, yel-

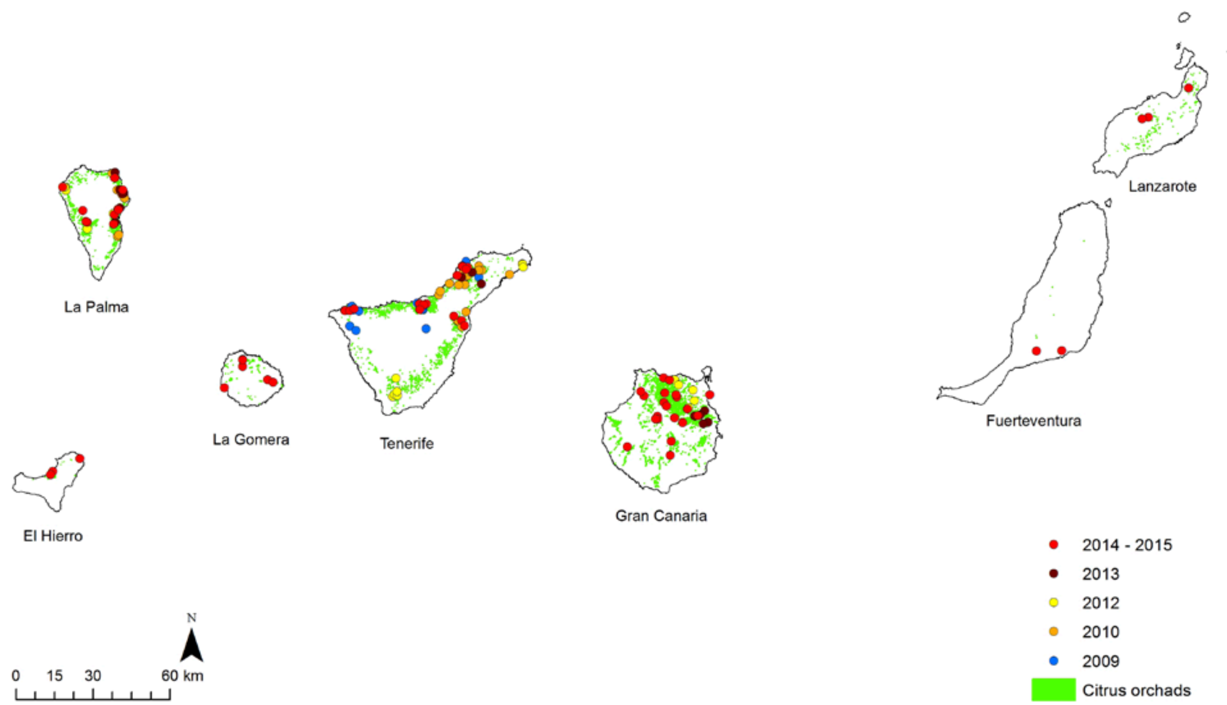

Figure 1. Canary Islands map with indication of areas with citrus cultivation, locations and years of surveys.

Table 1. Results of surveys conducted from 2009 to 2015 in Canary Islands and in 2014 and 2015 in Galicia, where *Trioza erytreae* is present.

Year	Locality	Inspected plots or sites	Surface accessed (ha)[a]	Inspected trees	Plots with *T. erytreae* symptoms	Trees sampled	Psyllids sampled	Real-time PCR results			
								Universal kit 'CaL' spp.[b]		'CaL' species-specific[c]	
								Plant material	Psyllids	Plant material	Psyllids
2009	Tenerife	21	21.6	22,026	20	821	510	2	0	0/2	nd
2010	Tenerife	20	13.4	20,791	20	819	286	3	0	0/3	nd
	La Palma	14	5.1	3,296	14	185	100	2	2	0/2	0/2
2011	La Palma	21	11.7	4,264	21	159	178	2	2	0/2	0/2
2012	Tenerife	9	14.2	2,079	9	172	0	2	nd	0/2	nd
	Gran Canaria	12	24.2	10,040	10	256	0	5	nd	0/5	nd
	La Palma	9	12.4	4,368	9	189	0	4	nd	0/4	nd
	Lanzarote	4	it[c]	17	0	17	0	0	nd	nd	nd
2013	Tenerife	3	0.3	48	3	32	100	1	3	0/1	0/3
	Gran Canaria	5	11.4	6,219	4	5	0	1	nd	0/1	nd
	La Palma	8	2.6	1,267	8	37	0	2	nd	0/2	nd
2014	Tenerife	15	17.7	9,445	15	165	100	0	0	nd	nd
	Fuerteventura	2	1.7	26	0	9	0	0	nd	nd	nd
	Gran Canaria	20	17.9	4,562	20	152	0	0	nd	nd	nd
	La Palma	10	6.7	2,910	10	101	0	0	nd	nd	nd
	Lanzarote	3	1.1	382	0	9	0	0	nd	nd	nd
	La Gomera	6	0.8	155	6	30	0	0	nd	nd	nd
	El Hierro	3	1.8	30	2	14	0	0	nd	nd	nd
	A Coruña	24	it	32	3	6	0	0	nd	nd	nd
	Pontevedra	68	it	88	14	28	45	0	0	nd	nd
2015	Tenerife	8	12.6	1,998	8	104	50	3	0	0/3	nd
	Gran Canaria	8	16	11,642	7	24	80	1	0	0/1	nd
	La Palma	4	5.5	2,285	4	40	50	0	1	nd	0/1
	La Gomera	3	0.5	228	3	9	10	0	0	nd	nd
	El Hierro	2	0.4	192	2	6	0	0	nd	nd	nd
	A Coruña	167	it	456	134	295	0	0	nd	nd	nd
	Pontevedra	715	it	2,245	491	1,612	60	2	1	0/2	0/1
Total		1,184	199.6	111,005	837	5,296	1,569	30	9	0/30	0/9

[a] it, individual trees.
[b] Bertolini *et al.*, 2014.
[c] Li *et al.*, 2006; Teresani *et al.*, 2014.

Figure 2. A, bumps on the upper leaf surface caused by *Trioza erytreae*; B, detail of these bumps; C, *T. erytreae* nymphs located on the underside of the leaf, D, eggs laid on the leaves; E, male of *T. erytreae*.

low shoots, typical blotchy mottled leaves or fruit colour inversion were never observed in the surveyed trees.

Thirty plant samples out of 3,355 sampled trees (0.9%) analyzed by real-time PCR using the universal '*Ca*. Liberibacter' spp. detection kit gave positive amplification. Similarly eight out of 1,464 (0.5%) squashed *T. erytreae* individuals amplified with the universal primers and probe gave positive reactions. However, none of these samples were positive with species-specific primers for any of the tested '*Ca*. Liberibacter' species. No amplification was obtained from controls from the kit or additional negative controls of plant material or psyllids that fed on leaves of healthy trees.

Northwest mainland Spain

In Galicia, the monitored areas covered the A Guardia and Rosal districts of Pontevedra province near the border with Portugal, through Pontevedra and A Coruña provinces along the coast, to Lugo province (Figure 3). The average precipitation of approx. 1,200 mm and summer temperatures of 20–22ºC ensure that this area has one of the mildest climates in Europe. *Trioza erytreae* associated symptoms were found continuously from the Portuguese border to Finisterre (A Coruña). The psyllid is widely distributed in all the coastal area of Pontevedra (from O Salnés in the North to O Baixo Miño in the South), and is advancing to inland areas (e.g., Caldas de Reis). In A Coruña the insect is present from O

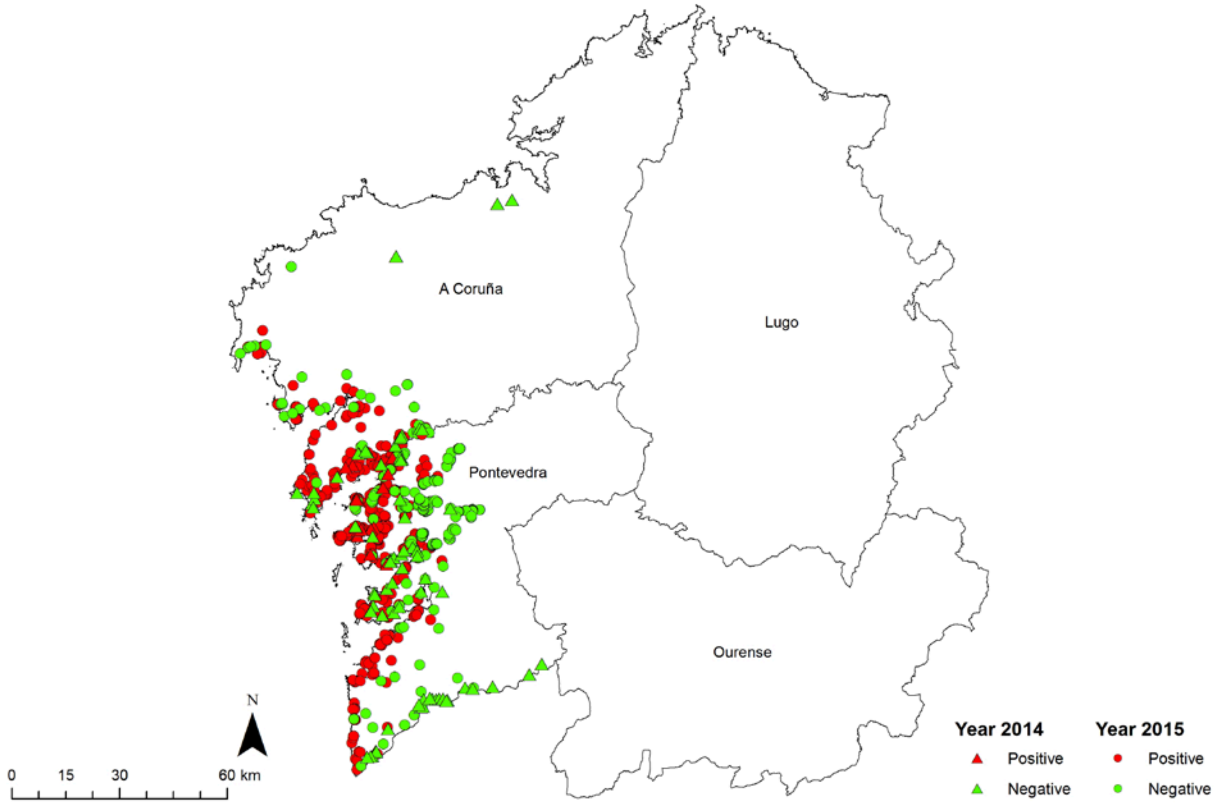

Figure 3. Galicia (Northwestern mainland Spain) map with the location of isolated trees sampled, years and detections of *Trioza erytreae* in the surveys.

Barbanza, in the South, to Muros-Noia and Fisterra, reaching coastal villages (Camariñas) and inland (Brión, Dodro, Padrón and Rois) (Figure 3). Symptoms of the pest were also found in trees grown in inland areas, where the insect spreads in summer, since it overwinters in protected trees along the coast. Sixty-six percent of the inspected trees showed *T. erytreae* symptoms, in many cases only on mature leaves of a few shoots showing nymphal bumps and nests. Mainly lemon trees were severely affected, but pest symptoms were also observed in mandarins, sweet and sour oranges, and grapefruit trees. In mid-February 2015 live male and female winged adults of *T. erytreae* were collected in Baiona (Pontevedra) on ornamental grapefruit trees. A total of 2,735 trees were monitored and surveyed. Table 1 shows the results from surveys conducted for two years (2014 and 2015). From the total inspected trees, 1,941 were sampled and analysed as well as 105 *T. erytreae* adult individuals. No typical yellow shoots, blotchy mottled leaves or fruit colour inversion were observed on monitored citrus trees grown in Galicia.

Two plant samples (0.1%) and one squashed *T. erytreae* individuals (0.9%) analysed by real-time PCR using the universal '*Ca*. Liberibacter spp' primers gave positive amplifications. None of these samples were positive with species-specific primers for bacterial HLB species or for '*Ca*. L. solanacearum'. No amplification was obtained from healthy controls.

Main Spanish citrus industry areas

Table 2 shows the results from surveys of HLB in the Huelva (southern Atlantic coast), Murcia, Castellón and Valencia (Mediterranean coast) provinces, where main plantations of citrus are present. Yellow sticky traps and the monitoring of trees indicated that either *T. erytreae* or *Diaphorina citri* were not present. A total of 324 plots representing 2,615 ha of citrus

Table 2. Surveys performed in Spanish main citrus production areas, where *Trioza erytreae* is absent.

Year	Locality	Inspected plots or sites	Surface accessed (ha)[a]	Inspected trees	Plots with *T. erytreae* symptoms	Trees sampled	Psyllids sampled	Real-time PCR results: Universal kit 'CaL' spp.[a]		'CaL' species-specific[b]	
								Plant material	Psyllids	Plant material	Psyllids
2010	Valencia and Castellón	150	165	66,000	0	91	0	1	0	0/1	nd
2011	Huelva	21	2,165	974,250	0	89	0	0	0	nd	nd
	Valencia and Castellón	150	165	66,000	0	42	0	1	0	0/1	nd
2012	Murcia	3	120	586	0	17	0	0	0	nd	nd
Total		324	2,615	1,106,836	0	239	0	2	0	0/2	nd

[a] Bertolini *et al*., 2014.
[b] Li *et al*., 2006; Teresani *et al*., 2014.

orchards were systematically inspected from 2010 to 2012, and 239 trees were sampled and analysed as indicated above. After analysis, two samples (0.8%) were amplified with universal primers and probe, but not with species-specific primers.

Discussion

Extensive monitoring of *T. erytreae* and a large survey of HLB were carried out, mainly in the Spanish areas where the African citrus psyllid vector of '*Ca*. Liberibacter' pathogens was detected, but also in the main citrus production areas of Spain. The surveys were followed by analyses based on tissue-print and squash real-time PCR according to the EPPO (2014) protocol for the detection of in plant material and insect vectors. A total of 111,005 trees were inspected, from citrus orchards in the Canary Islands and from isolated citrus trees, mainly lemon, in Galicia. The monitored trees represented approx. 15% of the total citrus industry of the Canary Islands (SECAGPA, 2012). Those monitored in Galicia represent approx. 3% of the officially estimated citrus trees grown in Pontevedra and A Coruña provinces (approx. 2% of the estimated citrus trees in Galicia) according to MAGRAMA (2015).

The high proportion of inspected plots showing pest symptoms (93%) in the Canary Islands compared with those in Galicia (66%) indicates that in northwest Spain the pest has entered recently, and is currently spreading. In some districts of A Coruña (Galicia) only new symptoms were observed in young leaves, while in the Canary Islands pest symptoms were easily found in mature leaves in all inspected trees. Although comparative studies on the impacts of *T. erytreae* damage on the different citrus species have not been carried out, lemon trees were the most affected citrus species, both in the Canary Islands and in northwest Spain, probably for the numerous and succulent shoots this species produces compared with sweet orange. Mandarin, grapefruit and other citrus species are not frequently cultivated in the surveyed areas. Nevertheless, some damage has been observed in these species in Galicia, and also in kumquat, *Murraya paniculata* and *Choysia ternata* (Pérez-Otero *et al*., 2016).

Although psyllid populations fluctuate, almost all the trees in the north of the five islands exhibited the characteristic damage caused by the African citrus psyllid, probably because high relative humidity and warm average temperatures are conditions conducive to rapid development of the insect (Bové, 2006).

There are differing opinions concerning the dispersal capabilities of *T. erytreae*. Catling (1973) stated that the African psyllid does not possess strong dispersal potential, and further stated that adults are not capable of sustained flights (Catling, 1978). In contrast, Samways and Manicom (1983) demonstrated that the insect is an excellent invader and can readily fly to areas of new vegetative flushes over distances of several hundred metres. The lack of recently published information about *T. erytreae* is surprising, in contrast to the abundance of studies about the other main vector, *D. citri* (Cocuzza et al., 2017). Fernandes and Aguiar (2001) suggested that the entry of *T. erytreae* in Madeira (Portugal) could be the result of clandestine plant material traffic probably brought from South Africa. The simultaneous appearance of *T. erytreae* in the western Canary Islands (Tenerife, La Palma, La Gomera and El Hierro), and more specifically along the northern coastal slopes of those islands, which are targets of the prevailing trade winds, suggests that natural airborne dissemination of the insects may have occurred from the nearby Madeira archipelago. All data gathered to date support this hypothesis, although an initial entry with infested plant material cannot be ruled out. Dissemination on infested plant material is more likely in the Gran Canaria island, where the insect was first observed as late as 2010 in the northern and central sector, and where it had spread throughout the island by 2013.

The entry of *T. erytreae* into Galicia could be from infested trees acquired in local markets in north Portugal, or in plants introduced from Africa. The establishment of the pest occurred in the very favourable micro-climatic area of Pontevedra province close to the coast. Since the first detection in mainland Spain in Vilanova de Arousa (Pontevedra) in 2014, the pest has mainly been found in lemon trees frequently grown in private and public gardens along the coast from A Guardia (Pontevedra) (Spain), near the border with Portugal, to the Ria de Muros (Rias Baixas). One year after the first detection, the pest was also found along the coast to Finisterre cape in A Coruña province progressing to the North. Detection and spread of *T. erytreae* thus occurred in a northern latitude never previously recorded. The same pattern of dissemination was observed some years ago with the brown citrus aphid *Toxoptera citricida*, considered the most efficient vector of *Citrus tristeza virus* (Hermoso de Mendoza *et al.*, 2008). Both pest vectors are currently spreading only in the north western area of Spain, far from the main citrus growing areas in the East and South of the Iberian Peninsula.

Another indirect result from the surveys reported here is the absence of *D. citri*, the Asian vector of the HLB pathogen, which was not been found in the inspections.

Therefore, to prevent spread of HLB, different plans and actions have been undertaken in Spain. Chemical control measures implemented over time in Madeira and in the Canary Islands have not been effective. The existence of thousands of isolated trees, many in private and public gardens throughout the largely rural landscapes of both archipelagos, make it extremely difficult to efficiently and sustainability contain *T. erytreae*. Biological control with *Tamarixia dryi* is an alternative to chemical control that has been shown to be effective in other islands (Aubert *et al.*, 1980; Etienne and Aubert, 1980). A programme for the introduction of this parasite should be considered as a possible strategy, although attention must be given to the presence of hyperparasites and other potential hosts for *T. dryi*. Measures taken in Spain include treatments with insecticides before pruning the shoots of affected citrus plants when the presence of the psyllid is confirmed, and the prohibition of traffic of susceptible plant material (except fruit and seeds) from the delimited areas.

Methods recommended by the EPPO diagnostic standard (EPPO, 2004) for detection of HLB-associated bacterial species were routinely used for the analysis, using direct systems of sample preparation (tissue-prints of plant material and squashing of psyllids). These methods greatly facilitated the collection of large numbers of samples in field conditions and rapid processing in the laboratory. A commercially available complete kit for the screening using universal '*Ca*. Liberibacter' spp. primers and a probe proposed by Bertolini *et al.* (2014) were selected. This methodology saved time and expense, since in only one screening real-time PCR reaction numerous samples with no amplification were discarded. The other option was to use the three real-time species-specific reactions that are necessary to discard the involvement of '*Ca*. L. africanus', '*Ca.* L. *americanus*' or '*Ca*. L. *asiaticus*' in each sample. As it has been suggested that the presence of the bacteria associated with HLB in psyllid vectors can be identified long before symptom expression in plants becomes apparent (Manjunath *et al*., 2008), *T. erytreae* specimens were also analysed.

From 7,104 analyzed samples, only 43 (34 from plant material and nine psyllids) amplified with the universal primers and probe, representing 0.6% of the analysed samples. Less than 1% of plant and individual psyllids from both the Canary Islands and Galicia amplified woth the universal primers and probe. Following the flow diagramme of the EPPO (2014) protocol, and the instructions of the available kit, all the analysed samples were then tested and resulted negative with species-specific primers and probe for the three HLB-causing bacterial species as well as for '*Ca*. L. solanacearum'. These percentages are in agreement with Bertolini *et al.* (2014), who reported amplification in 1.9% of *T. erytreae* specimens from Canary Islands that then tested negative with the species-specific primers and probe. Consequently, the very low percentage of samples giving positive amplification not related to '*Ca*. Liberibacter' spp. was considered acceptable given the many advantages of this detection strategy. The second part of the analysis, after the screening test, is necessary to complete positive detection. This should avoid further false positive records as was reported in Algarve (Portugal) in 2015 (EPPO, 2016b). The Spanish results show that the number of positive amplifications, not related with HLB associated bacteria, was very low, and the utilization of the species-specific primers and probe can discard the presence of HLB related bacterial species.

According to the surveys performed from 2009 to 2015, the known HLB associated bacterial pathogens are not present in Spain, neither in the Canary Islands nor in Galicia, or in other areas in which the vector is not currently present. Typical disease symptoms have not been found, and analyses of plant samples and vector specimens did not to detect '*Ca*. Liberibacter' spp. Trade globalisation is increasing the risk of uncontrolled plant material exchanges between continents. There is urgent need for extensive surveys in the Euro-Mediterranean area to detect these citrus pathogens and prevent HLB damage. This disease could seriously endanger the entire citrus industry of this region.

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