Letter to the Editor

ADDRESSING THE THREAT OF HUANGLONGBING IN THE MEDITERRANEAN REGION: A CHALLENGE TO SAVE THE CITRUS INDUSTRY

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SUMMARY

Even though Citrus huanglongbing (HLB), caused by *Candidatus* Liberibacter spp., is not yet present in the Mediterranean basin, this devastating disease is threatening the very survival of citrus in most parts of the world. In the framework of an international course on "Emerging and Quarantine Diseases of Mediterranean Fruit and Vegetable Crops", the participants were asked to hold group discussions to define measures for prevention and management of HLB in the Mediterranean Region. Following an introduction on the present situation of HLB in the world and emphasizing the facts that: (i) the African citrus psyllid, *Trioza erytrae*, is already present in the Canary and Madeira islands and threatens the western Mediterranean area and (ii) Asian HLB, with *Candidatus* Liberibacter asiaticus and the Asian citrus psyllid, *Diaphorina citri*, are present in Iran and the Arabian Peninsula and threaten the eastern Mediterranean area, a general discussion defined: (i) measures for prevention, including awareness, monitoring and surveillance, pest risk analysis, the establishment of quarantine measures, and practicing PCR detection methods for liberibacters in citrus and psyllids; (ii) action plans taking into consideration the presence of insect vectors and/or HLB-like symptoms; and (iii) future actions that must include the development of genetically modified citrus cultivars resistant to the causal liberibacter agents or to infection by the psyllid vectors. As management of HLB would be difficult in the Mediterranean area, in particular because of the small size of most citrus orchards, all efforts should be concentrated towards preventing HLB entrance and spread in the area.

Key words: *Candidatus* Liberibacter, pest risk analysis, citrus psyllid, HLB management

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INTRODUCTION

The Instituto Agronómico Mediterráneo de Zaragoza – Centre International de Hautes Études Agronomiques Mediterranées (IAMZ-CIHEAM) has held a course on "Emerging and Quarantine Diseases of Mediterranean Fruit and Vegetable Crops" from March 31 to April 5, 2014. Since emerging and re-emerging diseases are a risk for present and future agricultural productions, the objectives of the course were to create awareness about the spread of certain pathogens across international boundaries and to identify risk factors, while learning how to proceed in terms of prevention and control.

The course was organized through a series of lectures given by international experts (see "Acknowledgements"). The subjects covered legal and institutional issues as well as practical aspects, such as risk analysis and impact, diagnosis, epidemiology, prevention and control. Following these basic concepts, a series of case studies were illustrated on Mediterranean emerging and quarantine diseases caused by: (i) fungi (*Verticillium* in olive trees; *Monilinia fructicola* in stone and pome fruit trees); (ii) bacteria (*Acidovorax citrulli* in watermelon; *Candidatus* Liberibacter species in citrus); (iii) virus and virus-like agents (*Citrus tristeza virus* in citrus; emerging tomato viruses); and (iv) nematodes (*Meloidogyne* species in vegetable crops).

As part of the course, the participants were asked to form three discussion groups in order to propose, in a final debate, the best measures for the prevention and/or control of Citrus huanglongbing (HLB) in the Mediterranean

basin. They were asked to perform as representatives of their respective countries (Albania, Algeria, Egypt, France, Morocco, Spain, Tunisia and Turkey), so as to provide a good background on the characteristics of their Greater Regions (Southern Europe, North Africa, Middle East). Their proposals on HLB prevention and/or management in the Mediterranean Region are given hereafter, following some background information on the disease.

Background information on Huanglongbing. Several reviews on HLB have been published (Zhao, 1981; da Graça, 1991; Bové, 2006; Gottwald, 2010; Wang and Trivedi, 2013). HLB means "yellow shoot disease" in the Chaozhou dialect of southern China. This name was used by K.H. Lin in his report on HLB transmission by graft inoculation, a piece of work that demonstrated for the first time its infectious nature (Lin, 1956).

HLB is the most devastating disease that is currently threatening the very survival of citrus the world over. In addition to the yellow color of young shoots, the leaves show blotchy mottling, yellow veins and mineral deficiency symptoms, like that induced by zinc in particular. Fruits are lopsided, with color inversion and aborted seeds (Bové, 2006). So far, seed transmission of HLB has not been demonstrated. Eventually, affected trees decline and become uneconomical.

HLB symptoms were observed and accurately described for the first time in Asia, probably in the Punjab of today's Pakistan in 1927 by Husain and Nath (1927), and in southern China in 1943 by Chen Q. Bao (1943), in South Africa in 1928, in South America in 2004 (São Paulo State, Brazil), in North America in 2005 (Florida, USA) and in Central America in 2009 (Belize). The disease is now spreading throughout most citrus-growing areas of the world, only Australia, New Zealand, New Caledonia and the Mediterranean Basin being still HLB-free.

The aetiology of HLB is quite complex, as it involves three Gram-negative, phloem-restricted bacterial species: *Candidatus* Liberibacter asiaticus (Las) in Asia and America (since 2004), *Candidatus* Liberibacter africanus (Laf) in Africa and *Candidatus* Liberibacter americanus (Lam) in Brazil since 2004 (Bové, 2006). The prefix word "*Candidatus*" indicates that these bacteria have not been grown in axenic culture but were only characterized on a molecular basis. The three liberibacter species are transmitted in nature by two psyllid vectors: Las and Lam by *Diaphorina citri*, the Asian citrus psyllid, Laf by *Trioza erytreae*, the African citrus psyllid. Experimentally, however, *D. citri* transmits also Laf, and *T. erytreae* also Las (Bové, 2006).

Trees showing characteristic HLB symptoms may not be infected by liberibacters but by phytoplasmas such as a "16Sr Group IX phytoplasma" in the Brazilian states of São Paulo and Bahia (Teixeira *et al.*, 2008; N.A. Wulff, personal communication) and a "16Sr Group I phytoplasma" in China (Chen *et al.*, 2008), both of which have recently been reported also from citrus in Mexico. In São Paulo state (SPS) the vector of the 16Sr Group IX phytoplasma is most probably *Scaphytopius marginelineatus*, a leafhopper frequently found in sweet orange orchards (N.A. Wulff, personal communication).

All commercial citrus species are susceptible to HLB, regardless of the rootstock, and no curative approaches are available. Therefore, preventive measures are essential to avoid the entrance of the vectors and liberibacters in HLB-free areas. In those already affected by HLB, management strategies have been attempted based on three complementary measures: (i) reduction of the psyllid population by treating with insecticides several times a year; (ii) repeated removal (several times a year) of symptomatic trees as they are inoculum sources from which the phloem-feeding psyllid vectors acquire the liberibacters; (iii) replacement of the removed trees by healthy ones grown in covered, insect-free structures.

This "three-pronged-system" of HLB management has successfully been applied in large SPS farms (≥ 400 ha), resulting in more than 200,000 ha of citrus groves with less than 1% HLB-affected trees (Bové, 2012). Fundecitrus (Fundo de Defesa da Citricultura, Araraquara, SPS, Brazil) has conducted an evaluation of both well- and poorly-managed farms for determining the key factors associated with success or failure of HLB control (Belasque *et al.*, 2010). Such factors are: (i) HLB incidence in the region where the farm is located; (ii) percentage of HLB-affected trees when HLB-management is initiated; (iii) distance between the farm being evaluated and the "no-management" farms; (iv) size and shape of the farm; and (v) age of the trees.

The three-pronged system of HLB management, successful in SPS because of the many large farms with good know-how, would be difficult, if not impossible to implement in the Mediterranean basin where small citrus farms prevail. A solution to overcome this problem might come from the establishment of Citrus Health Management Areas (CHMA) where HLB management could be applied simultaneously to all citrus farms in the area, each farm being considered more or less as a block of a large farm. Finally, it has been experienced that many farmers are reluctant to remove trees that show incipient symptoms but still bear fruits. Such trees are sources of liberibacter inoculum and must be eliminated, for chemical control of psyllids without symptomatic tree removal is insufficient to control HLB (Timmer *et al.*, 2011; Timmer, 2014). In Florida, to avoid removing symptomatic trees, growers have used nutrient sprays to keep trees in production. Even though trees treated in this way show less mineral deficiency symptoms and look better, it remains controversial whether their yields increase. Notwithstanding a strong psyllid control, the percentage of infected trees in the nutrient-sprayed orchards has dramatically increased. In Brazil, nutrient sprays over a three-year period have had no beneficial effects.

The Mediterranean sea is bordered by European (Portugal, Spain, British Gibraltar, France, Monaco, Italy, Malta, Slovenia, Croatia, Bosnia-Herzegovina, Montenegro, Albania, Macedonia, Greece, Western Turkey and Cyprus), West Asian (Eastern Turkey, Syria, Lebanon, Israel and Palestine) and North African (Morocco, Algeria, Tunisia, Libya and Egypt) countries all of which grow citrus. These, and some additional neighboring citrus-growing countries (Jordan, Iraq, Iran, Sudan) have specific legislations. Nine countries of the area (Cyprus, Egypt, Greece, Israel, Italy, Morocco, Spain, Tunisia and Turkey) account for a 95% of the overall citrus production, whereas the remaining countries grow citrus only in small orchards or as backyard trees. Nonetheless, these minor producers are equally threatened by HLB and can become responsible for the entrance of the disease into the Mediterranean area. It should be kept in mind that: (i) the psyllid *Trioza erytrae* is already present in the Canary islands (Gonzalez Hernandez, 2003) and Madeira, which belong to Spain and Portugal, respectively; (ii) HLB threat approaches the Mediterranean basin from the South, for *Candidatus* Liberibacter africanus occurs in Saudi Arabia, Yemen and Ethiopia, Somalia and the Central African Republic and its vector (*T. erytrae*) in Saudi Arabia, Yemen, Ethiopia, Erythrea, Somalia and Sudan, whereas *Candidatus* Liberibacter asiaticus has been recorded from Saudi-Arabia, Yemen and Iran, and its vector (*Diaphorina citri*) from the same countries plus Oman and the United Arab Emirates.

PROPOSALS FOR HLB PREVENTION AND MANAGEMENT

The following measures for prevention and management of HLB in the Mediterranean Region have been proposed by the Course participants after group discussions and a final debate.

Measures for prevention. *Awareness.* Prevention measures are key issues to avoid or delay the entrance of the HLB liberibacters and their psyllid vectors in the Region. It is critical to build up awareness on the importance of the disease and its devastating effects to all commercial citrus species and cultivars, including citrus relatives used as ornamentals. Awareness must reach: (i) farmers, nurserymen, extension and plant protection services and the overall citrus industry (fruit production and processing, including trade of ornamental citrus and citrus relatives); (ii) the population at large. An effort must be made to involve governmental institutions such as Ministries of Agriculture, Research Institutes for Horticulture and Plant Protection, consultancy agencies, germplasm collections, food safety agencies, etc. Financial resources must be made available to promote the creation of a specialized task-force responsible for contingency plans and awareness campaigns by all possible types of media: internet, telecommunication, newspapers, radio and television. Posters or stickers containing information on psyllid vectors and HLB symptoms should be installed at high-traffic places (e.g. toll-stations on freeways, supermarket entrances) and, why not, on the back of the cars, for capturing the attention of pedestrians and car drivers. Special attention should be paid to international airports and ports. Airplane and ship companies must be encouraged or forced to announce that it is prohibited to carry and introduce citrus propagating or planting material from a foreign country. All plant materials must be declared at customs and quarantine must be reinforced. These awareness measures should be discussed, improved and implemented in the frame of a large collaboration among the countries. The setting up of a Mediterranean HLB Committee with representatives of each country is highly desirable.

Monitoring and surveillance. A contingency plan should be launched promptly encompassing: (i) adequate quarantine measures; (ii) implementation of research activities focussing on the development/availability of detection tools. PCR-primers for the detection of Laf, Las, Lam, 16Sr group IX and 16Sr group I phytoplasmas, as well DNA samples of these liberibacters and phytoplasmas should be made available to accredited laboratories of each of the countries of the Region. This will make it possible to check for the above agents both in suspicious citrus leaves and psyllid vectors. As it would be highly desirable to build collaborative approaches among all the countries at risk so as to operate in a coordinated and transparent manner, specialists must be trained for acquiring adequate knowledge on: (i) HLB vectors and their identification; (ii) characteristic HLB leaf and fruit symptoms in various citrus cultivars (orange, mandarin/clementine, grapefruit, lemon/lime), citrus relatives (e.g. *Murraya paniculata*) and rootstocks; and (iii) the situation regarding HLB and its vectors in neighboring countries. This requires that the citrus-growing countries in the Region work in a coordinated manner, following essentially the same surveillance rules and using standardized, validated and verified methods.

A country free of HLB and HLB vectors is at risk if a neighboring country is affected. For instance the presence of *T. erytreae* in Madeira and Canary islands is a risk factor for western Mediterranean countries such as Morocco, Portugal and Spain, which entertain close relationships with these islands, with which they are linked by sea and air. Therefore, within areas close to ports and airports, or on the roads at land borders, special surveillance, possibly with sticky traps for insects, is recommended. Search for putative HLB symptoms should also be regularly carried out in the risky areas.

Pest risk analysis (considering a pest as any species, strain or biotype of plant, animal, or pathogenic agent, injurious to plants or plant products, according to the

International Plant Protection Convention, IPPC)**.** Plant health authorities from countries in the Mediterranean basin should be concerned about the risks associated with the introduction of HLB in the area. The pests (liberibacters and vectors) are of enough quarantine concern to be considered for risk analysis in the area, taking into consideration: (i) the pathways present and potential pest hazards; (ii) the pest that may require phytosanitary measures; and (iii) that it is essential to revise the phytosanitary policies and priorities.

Different organizations like the European Food Safety Authority (EFSA) or the European and Mediterranean Plant Protection Organization (EPPO) are specialized in performing "Pest Risk Analysis" (PRA), which encompasses three actions:

(i) the initiation point in which pests and pathways of concern and the PRA area are identified. Relevant information is collected and pests are identified as possible candidates for phytosanitary measures, either individually or in association with a pathway;

(ii) the process for PRA which is undertaken following three interrelated steps: (a) pest categorization, (b) assessment of the probability of introduction and spread, and (c) assessment of potential economic consequences (including environmental impacts). As a result of this process, the pest may be considered appropriate for PRA. For each pest, all or part of the PRA area may be identified as an endangered area. A quantitative or qualitative estimate of the probability of introduction of a pest, and the corresponding quantitative or qualitative estimate of possible economic consequences (including environmental consequences), must be obtained and documented or an overall rating must be assigned. These estimates, with the associated uncertainties, are utilized in the pest risk management stage.

(iii) Pest risk management in which the conclusions achieved are used to decide whether risk management is required and the strength of measures to be used. Pest risk management options should be identified, taking into account the degree of uncertainty in the assessment of economic consequences, probability of introduction, and the respective technical justification of those options. The result of a PRA procedure will be either that no measures are identified which are considered appropriate, or that one or more management options that have been found to lower the risk associated with the pest(s) to an acceptable level are selected. These management options form the basis of phytosanitary regulations or requirements.

Quarantine measures. Quarantine measures should be enforced for adequately controlling the imports of propagation material (plants for planting, budwood, etc.) of citrus and other rutaceous plants when permitted, being aware that not only commercial citrus cultivars, but also ornamental rutaceous species are to be considered. All propagation material to be imported must be certified free of HLB and re-confirmed free of HLB (and vectors) on arrival. Importations from HLB-affected countries must be avoided. Unfortunately, the quarantine measures and contingency plans in the Mediterranean area will face the difficulties inherent to the specific legislations of the many countries in the Region. Harmonization of legislation and related measures is highly recommended.

Action plans. In many cases psyllid vectors (*T. erytreae* or *D. citri*) have been detected or were present in a country before HLB occurred. In South Africa, *T. erytreae* was present since 1897 but HLB symptoms were observed for the first time only in 1928. As to *D. citri*, the insect was reported in Brazil in 1942 and HLB in 2004; for Argentina the years were 1984 and 2012; for Florida: 1998 and 2005, for Guadeloupe (France): 1998 and 2012, for Texas: 2001 and 2012, for California: 2008 and 2012. In Madeira (Portugal) and Canary Islands (Spain) the presence of *T. erytreae* has been ascertained since 1994 and 2002, respectively, but as of August 2014, HLB has not been observed.

The most critical situation occurs when, in the presence of insect vectors, trees showing HLB-like symptoms have just been identified, as it was the case of Brazil in 2004 and Florida in 2005. Under these conditions, an action plan should be enforced immediately. It must be confirmed that the HLB-like symptoms are indeed associated with the presence of either one of the three liberibacters (Laf, Las or Lam), one of the two phytoplasmas (16Sr group I and 16Sr group IX), or a new bacterial agent. The use of reliable generic and specific detection tools, such as conventional PCR, real-time PCR, etc. is highly recommended. To avoid misinterpretations, the results of this first approach should be discussed with HLB experts.

If HLB-like symptoms are observed in an area free of HLB vectors, the above actions must also be undertaken. But, of course, HLB spread will be highly dependent on the presence of insect vectors, psyllids or leafhoppers, in the area where HLB is first detected.

Following the first HLB detection in a citrus plot of a region hitherto disease-free a thorough monitoring should be carried out by: (i) careful checking for additional HLB-affected trees within the initial plot and surrounding areas; (ii) tracing the source of the HLB-affected trees (legally imported or propagated in a local nursery; country of origin if imported; illegal introduction); (iii) search for and identification of putative vectors in the area.

If citrus psyllids are known to be present or have just been identified, frequent and intensive insecticide treatments should be carried out to restrain the psyllid population. All infected plants should be immediately removed and adequately disposed of (transported and destroyed), but the HLB strain involved should be kept for further work by graft-propagating buds or budwood sticks from the affected tree(s) on appropriate citrus rootstocks under quarantine conditions. The initially affected plot and the

adjacent orchards should be periodically surveyed for the presence of additional symptomatic trees. Indeed, it should be kept in mind that one of the major difficulties for HLB survey comes from the fact that all infected trees in an orchard do not show symptoms at the same time. This is because there is a latency period for symptom expression: the time required for an infected tree to become symptomatic (latency period) varies from *ca.* 6 to *ca.* 18 months. This is one of the reasons why several surveys per year are required to identify as many infected trees as possible.

It should be kept in mind that the above actions will only be successful if competent authorities have already issued legislative provisions to face the situation and financial resources are available to compensate the growers for the removal of symptomatic trees from their orchards. In addition, strict quarantine measures should be established to isolate the affected area from yet non-affected areas.

As indicated above, when the presence of HLB-affected trees is associated with the presence of psyllid vectors, intensive insecticide treatments should be applied in a compulsory way. However, it should be remembered that insecticide treatments which will lower the insect population will not achieve the desired effects unless the source of inoculum is also reduced by periodical removal of HLB-affected trees. In Brazil, in the farms where these measures have been applied, it has been possible to keep the percentage of removed trees below 1% per year, while in the absence of HLB management, the percentage of trees to be removed increased rapidly, from a low percentage to over 20% within only three years.

Future actions. At this moment, a time when HLB is not (yet) present in the Mediterranean area, everything must be done, through a coordinated effort among all the partners and countries, to prevent its introduction. Indeed, once the disease and its vector(s) have been detected, control of HLB, as it is known today, will be most difficult. As mentioned, HLB management through the three-pronged-system was successful in Brazil, where citrus is grown in very large farms and control started at a very early phase. On the contrary, in Mediterranean countries, farms are much smaller and the three-pronged-system might not meet with success unless, as suggested, Citrus Health Management Areas (CHMA) are established, in which each small farm is considered as a block of a large farm where insecticide sprays but also removal of symptomatic trees are applied to all the blocks within the shortest time possible. CHMAs have been organized in Florida only for insect control, but with no tree removal, and have not been successful as far as HLB control is concerned. For these reasons, when HLB-affected trees are identified for the first time in an area, in particular when a psyllid vector is already present in the area, attempts to eradicate the disease become an obligation. In general, at least four inspections per year are required to identify and remove symptomatic trees. The number of trees removed per year must carefully be recorded. When HLB management is successful, the number of trees removed per year should decrease from one year to the next. Removed trees must be recorded on the orchard map so as to see where and where from infection occurs. Autumn and winter are better seasons than spring and summer to see HLB symptoms. Hence, the number of symptomatic trees removed in winter might be higher than in summer, a situation that should not be misinterpreted as meaning that the HLB severity has increased. Only the total number of trees removed per year should be compared (Belasque *et al*., 2010).

The current HLB management strategy is regarded today only as a measure to gain time for new HLB control systems to become available. Most of these systems involve the development of citrus cultivars resistant to the causal agents or to infection by the psyllid vectors. In spite of many efforts, no natural resistance has been found within citrus and citrus relatives; therefore resistance has to come from genetically-modified cultivars. Citrus varieties expressing genes for antibacterial peptide have already been obtained and are tested for HLB-resistance in the field, but none has so far been used in commercial orchards. Genes for resistance can be introduced into the citrus genome by Agrobacterium-mediated transformation (Cervera *et al*., 2005; Marques, 2015), but also by infection of citrus plants by a viral vector. Such a vector, based on a local strain of *Citrus tristeza virus* (CTV) has been developed in Florida by W. Dawson and co-workers (Folimonov *et al*., 2007; El-Mohtar and Dawson, 2014). Viral vectors for areas other than Florida, say a Mediterranean country, must be developed using CTV stains infecting citrus in that country. Finally, as HLB is not present in the Mediterranean area, the development of HLB-resistant cultivars for this area has to be done in collaboration with competent laboratories, obviously from countries where HLB is present.

Coordination among citrus-growing countries is a key issue to win the battle against HLB. A task force is essential to undertake a number of actions that must involve: (i) information to growers and pertinent national agencies; (ii) development of joint research programs. Such projects must involve horizontal approaches with the input of entomologists, plant pathologists, plant breeders and other plant scientists, and the involvement of public and private agencies. In addition, a PRA should be requested to European competent agencies for official support of the initiatives to be undertaken.

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