1. Content of the 'Topic Description' document

1.1 Topic area

Diagnostics, field detection, surveillance.

1.2 Topic title

Comparison of real-time PCR detection methods for the plant pathogen '*Candidatus* Liberibacter' spp. causing the Huanglongbing disease on *Citrus* spp.

1.3 Description of the problem the research should solve

The Huanglongbing (HLB / Citrus greening) disease is associated with three species of phloem restricted bacteria recognized as members of '*Candidatus* Liberibacter', namely 'asiaticus', 'americanus', and 'africanus'. Although the causal agents of the disease have not been cultivated in axenic medium yet, they represent one of the most destructive and wide-spread diseases of citrus across Asia, Africa, and America. It is mainly affecting citrus species, cultivars and hybrids and some other hosts within the *Rutaceae* family. This disease is associated with two main psyllids: *Diaphorina citri* and *Trioza erytreae*, and is also transmitted by propagative plant material, which can spread outbreaks on great distances.

As part of an initial diagnosis, the visual inspection of symptomatic plants is a routine method for the surveillance of HLB disease, but it can be misinterpreted. Yellow shoots, leaf blotchy mottle, and lopsided fruits with colour inversion and aborted seeds are typical symptoms on HLB affected trees. However, symptoms alone are not enough to complete a diagnosis, as they can be confused with nutritional disorders (zinc, iron, manganese deficiencies) or with other diseases (*Citrus tristeza virus*, Stubborn, citrus blight, Australian citrus dieback). These three *Liberibacte*r species responsible for the HLB can also be present in the host plant at a very low concentration and as the disease develops irregularly, individual trees may show a mixture of normal and diseased sectors.

Although conventional PCR is a sensitive and specific method, the PCR tests can lead to false negative results due to the low titter and uneven distribution of the bacterium in the host plants, especially at early stage of the infection (Jagoureix *et al.*, 1994)[1]. Hence, conventional PCR method is not recommended for the detection of *Ca*. Liberibacter spp. responsible for the HLB disease in symptomless plants (Li W. *et al*., 2006)[2]. Being more sensitive, real-time PCR tests may be useful in programs for the production of certified citrus nursery trees and in post-entry quarantine and is more adapted for early detection.

Various diagnostic real-time PCR tests were published and assessed for their performance, but with different procedures. Comparison of these protocols through the same procedure is hence required in order to fully compare the performance of these tests. The three main real-time PCR tests published that are routinely used are those from Bertolini, *et al*. (2014)[3]; Li W. *et al*. (2006)[2]; and Morgan *et al*. (2012)[4]. Moreover, a real time LAMP has been successfully developed for testing '*Candidatus* Liberibacter asiaticus' in the insect vectors (Keremane *et al*., 2015)[5]. Its use in plant material has also been tested but not validated. This method can be applied on site using a real time LAMP device, accompanied with an innovative extraction simplifying the diagnostic method (Yaseen *et al.*, 2015)[6].

The recent suspicious case of HLB declared by the Portuguese authorities and the absence of confirmation, underlines the need for a thorough and comparable assessment of detection tests to guarantee the reliability of the results obtained.

Therefore, collaboration at an international level would be beneficial to compare these three main real-time PCR protocols for identification of *Ca*. Liberibacter spp. responsible for the HLB disease in both symptom and symptomless plants of *Citrus* spp.

1.4 Description of the expected results

1. Production of performance data

As a prerequisite for the organisation of the collaborative tests performance study, an intra-laboratory assessment of the three above-mentioned real-time PCR tests will be achieved. This step will generate comparable performance data sets on each of the three real-time PCR tests, within a unique framework, following the European and Mediterranean Plant Protection Organization validation protocol (EPPO – PM7/098).

The assessment will involve the evaluation of the analytical specificity, sensitivity, and repeatability of the three real-time PCR protocols on DNA samples, extracted from fresh or dried tissues from symptomatic or asymptomatic diseased trees, or from disease-free trees.
Whereas the Bertolini, *et al*. (2014) and Li W. *et al*. (2006) real-time PCR can both detect the three species of '*Candidatus* Liberibacter' responsible for the HLB; the Morgan *et al*. (2012) real-time PCR is focusing on the sole identification of '*Candidatus* Liberibacter asiaticus'. Hence, those tests will be evaluated and compared for their respective targets, among several replicates, along with non-targets organisms.

The Bertolini, *et al*. (2014) real-time PCR is commercially available in a kit, and could be tested as a fourth real-time PCR modality to complement data performance of the three other tests.

Partners of this project are invited to provide material (DNA) for this preliminary work, in order to broaden and diversify the reference materials used for validation purpose. They will also be associated in the design of this assessment. The performance data obtained during the intra-laboratory evaluation will be collected through a report and will serve as a basis for comparison for the collaborative tests performance study.

In the same way, an intra-laboratory assessment of the LAMP test described previously will be achieved for the detection of '*Candidatus* Liberibacter *asiaticus*' in plant material. This step will generate also performance data sets following the European and Mediterranean Plant Protection Organization validation protocol (EPPO – PM7/098).

The assessment will involve the evaluation of the analytical specificity, sensitivity, and repeatability of the LAMP test on DNA samples, extracted from fresh or dried tissues from symptomatic or asymptomatic diseased trees, or from disease-free trees.

2. Collaborative tests performance study

This step will evaluate the inter-laboratory reproducibility of the three real-time PCR protocols and one real-time LAMP protocol through a collaborative test performance study. The possible effects of different operators, equipment or environments on the expected results will be assessed and will provide guidance for the laboratories, which are using or are intending to use these tests.

This step involves the cooperation of the partners' laboratories for analysing the samples within a pre-discussed time frame in order to build a trustful framework around this assessment. Among others, the results of this study will allow improving consensus diagnostic protocol already existing (e.g. EPPO diagnostic protocol PM7/121) or currently under preparation (e.g. IPPC protocol on '*Candidatus* Liberibacter' spp. on *Citrus* spp.). Collaborative work will help to harmonize operating procedures throughout routine

laboratories and to avoid trade issues. Additionally, the performance values that would be produced would help those laboratories applying for accreditation or accredited for such analysis, by providing them with minimum performance to be reached. This collaborative study and its results would also help the NPPO to design their survey or control policy by establishing the minimum level of detection that could be detected in laboratories.

1.5 Beneficiaries of this research product

- National and EU policy makers
- National Plant Protection Organisations, including risk managers and diagnosticians
- EPPO and its members (validated diagnostic protocols; information contributing to EPPO PRAs)
- Citrus industry and other stakeholders

1.6 Research funders and research contribution/ distribution

Funding organisation	Research activity and researchers involved
1. French Agency for Food, Environmental and Occupational Health & Safety, France Geraldine Anthoine geraldine.anthoine@anses.fr	-Full assessment of the real-time PCR protocols. -Organization and participation to the collaborative study. Contact person: Gilles Cellier gilles.cellier@anses.fr
2. CIHEAM-Istituto Agronomico mediterraneo of Bari, Italy Anna Maria D'Onghia donghia@iamb.it	-Full assessment of the real-time LAMP protocol. -Participation in the organization of the collaborative study. -Participation to the collaborative study. Contact person: Thaer Yaseen y.thaer@iamb.it
3. National Institute for Agricultural and Veterinarian Research, Portugal Leonor Cruz leonor.cruz@iniav.pt	-Participation in the collaborative study of real-time PCR protocols. -DNA providers. Contact person: Eugénia de Andrade and Paula Sá Pereira eugenia.andrade@iniav.pt
4. National Institute for Agricultural and Food Research and Technology, Spain Anabel De La Peña anaisabel.delapena@inia.es	-Participate in the collaborative study. Contact person: Jaime Cubero cubero@inia.es
5. Department of Agriculture, Animal and Plant Health Inspection Service, United States of America Laurene Levy laurene.levy@aphis.usda.gov	-Participate in assessment of real-time PCR methods. Contact person: John Rascoe john.rascoe@aphis.usda.gov
6. Biological Control Research Institute, Turkey Elen Ince	-Participation in the collaborative study. Contact person: Elen Ince elen.ince@tarim.gov.tr

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1.7 Research project partnership outside Euphresco

Euphresco funding ensures a certain level of transnational collaboration among Euphresco member countries. It is possible, if the funding consortium is interested, to contact funding organisations or research groups outside the geographical area covered by Euphresco members. The Euphresco coordinator could advertise the research topic in order to have an enlarged collaboration. If funders are interested in this possibility, please check the case below:

☒ *The funding consortium of the topic mentioned in section 1.6 requires to advertise the topic outside the Euphresco network*

Partnerships outside the Euphresco framework are foreseen with other laboratories, which are using real time PCR as a routine, in order to provide a critical mass of data to be interpreted for the collaborative tests performance.

1.8 Any other relevant information on content

The work produced among all laboratories could be valorised through communication to international congresses, such as the "*International Research Conference on Huanglongbing*" (Florida - USA) in 2019. Also, publication in a high standard peer-reviewed scientific journal would be considered.

References

1. Jagoueix S, Bove JM, Garnier M. The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the Proteobacteria. Int J Syst Bacteriol. 1994; 44: 379-386.

2. Li W, Hartung JS, Levy L. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. J Microbiol Methods. 2006; 66: 104-115.

3. Bertolini E, Felipe RTA, Sauer AV, Lopes SA, Arilla A, Vidal E, Mourão Filho FAA, Nunes WMC, Bové JM, López MM, Cambra M. Tissue-print and squash real-time PCR for direct detection of '*Candidatus* Liberibacter' species in citrus plants and psyllid vectors. Plant Pathol. 2014; 63: 1149-1158.

4. Morgan JK, Zhou L, Li W, Shatters RG, Keremane M, Duan YP. Improved real-time PCR detection of '*Candidatus* Liberibacter *asiaticus*' from citrus and psyllid hosts by targeting the intragenic tandem-repeats of its prophage genes. Mol Cell Probes. 2012; 26: 90-98.

5. Manjunath L. Keremane, Chandrika Ramadugu, Esteban Rodriguez, Ryo Kubota, Scott Shibata, David G. Hall, Mikeal L. Roose, Daniel Jenkins, Richard F. Lee. A rapid field detection system for citrus huanglongbing associated '*Candidatus* Liberibacter asiaticus' from the psyllid vector, Diaphorina citri Kuwayama and its implications in disease management, Crop Protection, 2015; Volume 68, 41-48,

6. Yaseen T., Drago S., Valentini F., Elbeaino F., Stampone G. and A. M. D'onghia. On-site detection of *Xylella fastidiosa* in olive trees (Olea europaea L.) and insects using the real-time loop-mediated isothermal amplification method. Phytopathologia Mediterranea, 2015, 54, 1, temp17−25 DOI: 10.14602.

2 Euphresco management aspects of the project

2.1 Indication of the topic budget

Funding organisation [a]	Mechanism [b]	Total Budget [c]
1. Anses (FR)	NC	€ 100 000
2. CIHEAM (IT)	NC	€ 20 000
3. INIAV (PT)	NC	€ 5 000
4. INIA (ES)	NC	€ 30 000
5. APHIS (USA)	NC	€ 4 663
6. BCRI (TK)	NC	€ 15 000
total		€

2.2 Expected duration of the project (only for non-competitive topics)

32 months (September 2016 - April 2019).

2.3 Any other relevant information on topic organisation and management

[a] First member is project coordinator. A minimum of two partners are necessary for each proposal. Add lines as needed.

[b] Please indicate the preferred mechanism (e.g. real pot RP; virtual pot VP; non-competitive NC), or several mechanisms if there is flexibility.

[c] Optional, as this amount can still change in the next phase. In-kind contribution should also be indicated in this column.