Technical documentation on Candidatus Liberibacter Solanacearum

# Introduction: Candidatus Liberibacter Solanacearum (CLso)

1. Candidatus Liberibacter solanacearum (CLso or Lso) is a Gram-negative bacterium, belonging to the class of Alphaproteobacteria which is currently not cultivable in vitro. It is described as a phloem-limited plant pathogen capable of causing symptoms on Solanaceae (Zebra chip disease on potato) or Apiaceae crops such as potato, tomato, bell pepper, tobacco, celery or carrot.
2. The bacterium is described as essentially transmitted by psyllids, insects belonging to the Psyllidae family. The host range of the bacteria is limited by the host range of the insect vector, which is known to be restricted to a few phylogenetically related plants (Haapalainen 2014). The studies by Munyaneza et al. (2016) and Antolinez et al. (2017) also show that the risks of cross-contamination between potatoes and carrots are insignificant.
3. On apiaceae, symptoms are characterized by uncontrolled development at the crown, curling and/or discoloration (yellow, bronze to purple) of the leaves, stunting of the main root and proliferation of secondary roots. These are symptoms that can be compared to other diseases: carrot psyllid, aster yellows (Aster Yellows phytoplasma) or Spiroplasma citri (Munyaneza, 2012).
4. CLso bacteria are divided into nine haplotypes - A, B, C, D, E, F, G, H, and U (Haapalainen et al. 2020). Haplotyping is based on the presence of mutations in certain genes. Haplotypes differ depending on the host, vector and geographic location. The haplotypes [C], [D], [E] and [U] have been identified on apiaceae and localized in Europe. Haplotypes [A], [B] and [F] have been identified on nightshades, reported from the USA and New Zealand. The haplotypes identified in France are D and E (Hajri et al., 2017).
5. In recent years, in recent years, certain countries have taken measures against CLso which have strongly impacted the imports of carrot seeds from France. Yet, since 2017, several studies have been able to state that transmission of the bacteria from the seed to the food crop is not proven. Thus, the authorities of the importing countries no longer have rational scientific arguments to prohibit the introduction of carrot seeds.

# Background study

Since 2015, several studies have been carried out on the transmission of the bacterium Candidatus Liberibacteur using carrot seeds:

* In 2015, the publication Bertolini et al. (2015) concluded that Lso could be transmitted from seeds to carrot plants.

This publication resulted in the implementation of strict measures on imports of carrot seeds from certain countries in Europe, including France.

Subsequently, several studies have provided new scientific evidence that the bacteria are not transmitted by carrot seeds**:**

* The French Agency for Food, Environmental and Occupational Health and Safety (ANSES) “Agence Nationale Sécurité Sanitaire Alimentaire Nationale“ in France, has carried out an extensive work on the Lso: the results of two studies by Loiseau et al. 2017a and b failed to demonstrate Lso transmission from seed to plants and showed that seed is not a vector of the disease.
* Several studies by Japanese researchers also argue that transmission of the bacteria through semen is unlikely.
* The Israeli study, Mawassi et al. (2018), the Finnish study, Haapalainen, M. et al. (2018) as well as the Italian study, Carminati et al. (2019), also obtained similar results concluding the non-transmission of CLso.

To summarize, today **7 studies**, carried out by different and independent organizations, have been able to demonstrate **the absence of transmission** of the bacteria by carrot seeds and contradict the study by Bertolini et al. (2015), which is **the only study** which supports that CLso is **transmissible** by seed:

|  |  |  |
| --- | --- | --- |
| **Date** | **Etudes** | **Conclusion sur la transmission de CLso sur la semence** |
| 2015 | **Bertolini et al. (2015) :**  E. Bertolini; G. Teresani; M. Loiseau; F. Tanaka; S. Barbe; C. Martinez; P. Gentit; M. López y M. Cambra. 2015. Transmission of ‘Candidatus Liberibacter solanacearum’ in carrot seeds. PlantPathology 64 (2): 276-285, April 2015. First published: 21 May 2014.  Disponible sur :  <https://bsppjournals.onlinelibrary.wiley.com/doi/full/10.1111/ppa.12245> | Transmission |
| 2017 | **Loiseau, M. et al. (2017a) :**  M. Loiseau, I. Renaudin, P. Cousseau-Suhard, P.-M. Lucas, A. Forveille, and P. Gentit (ANSES). 2017. Lack of evidence of vertical transmission of 'Candidatus Liberibacter solanacearum' by carrot seeds suggests that seed is not a major transmission pathway. Plant Disease 101(12): 2104-2109, December 2017.  Disponible sur :  <https://apsjournals.apsnet.org/doi/10.1094/PDIS-04-17-0531-RE> | Pas de transmission |
| 2017 | **Loiseau, M., et al. (2017b) :**  M. Loiseau, I. Renaudin, P. Cousseau-Suhard, F. Poliakoff, P. Gentit. 2017. Transmission tests of 'Candidatus Liberibacter solanacearum' by carrot seeds. Acta Horticulturae (1153): 41-46, March 2017.  Disponible sur :  <https://www.ishs.org/ishs-article/1153_7> | Pas de transmission |
| 2017 | **Oishi et al. (2017) :**  M. Oishi, S. Hoshino, Y. Fujiwara, S. Ushiku, Y. Kobayashi, I. Namba. 2017. A comparison of protocols to detect Candidatus Liberibacter solanacearum from carrot seeds, research on the effectiveness of propidium monoazide treatment and evaluation of seed transmission in carrot seeds. Res Bull Plant Prot Japan 53:111–117, March 2017.  Disponible sur [en japonais] :  <https://www.maff.go.jp/pps/j/guidance/r_bulletin/pdf/rb053_016.pdf> | Pas de transmission |
| 2018 | **Haapalainen, M. et al. (2018) :**  M. Haapalainen, J. Wang, S. Latvala, M. T. Lehtonen, M. Pirhonen, and A. I. Nissinen. 2018. Genetic variation of 'Candidatus Liberibacter solanacearum' haplotype C and identification of a novel haplotype from Trioza urticae and stinging nettle. Phytopathology 108(8): 925-934, June 2018.  Disponible sur :  <https://apsjournals.apsnet.org/doi/10.1094/PHYTO-12-17-0410-R> | Pas de transmission |
| 2018 | **Mawassi et al. (2018)**  M. Mawassi, O. Dror, M. Bar-Joseph, A. Piasezky, J. M. Sjölund, N. Levitzky, N. Shoshana, L. Meslenin, S. Haviv, C. Porat, L. Katsir, S. Kontsedalov, M. Ghanim, E. Zelinger-Reichert et al. 2018. 'Candidatus Liberibacter solanacearum' Is Tightly Associated with Carrot Yellows Symptoms in Israel and Transmitted by the Prevalent Psyllid Vector Bactericera trigonica. Phytopathology 108 (9):1056-1066, September 2018.  Disponible sur :  <https://apsjournals.apsnet.org/doi/10.1094/PHYTO-10-17-0348-R> | Pas de transmission |
| 2019 | **Carminati et al. (2019)**  G. Carminati, E. Satta, S. Paltrinieri, A. Bertaccini. 2019. Simultaneous evaluation of ‘ Candidatus Phytoplasma’ and ‘Candidatus Liberibacter solanacearum’ seed transmission in carrot. Phytopathogenic Mollicutes. 9, 141–142, January 2019.  Disponible sur :  <http://www.indianjournals.com/ijor.aspx?target=ijor:mollicutes&volume=9&issue=1&article=071> | Pas de transmission |
| 2020 | **Fujikawa, T. et al. (2020) :**  T. Fujikawa, K. Yamamura, K. Osaki, N. Onozuka, M. Taguchi, A. Sasaki, M. Sato. 2020. Seed transmission of ‘Candidatus Liberibacter solanacearum’ is unlikely in carrot. Journal of General Plant Pathology 86:266–273, May 2020.  Disponible sur :  <https://link.springer.com/article/10.1007/s10327-020-00927-1> | Pas de transmission |



# Comparative studies

At the moment, the study by Bertolini et al. (2015) is **the only publication** that suggests transmission of Lso by carrot seeds. However, its results are controversial as they could not be confirmed by more recent studies:

1. Indeed, **the conclusions** of Bertolini et al. (2015) claim transmission of Lso from seed to carrot plant, however:
   1. The Loiseau et al. (2017b) concludes that transmission of Lso by seeds has not been confirmed, although the seed lots analyzed have the same origin as those used by Bertolini et al. (2015). The authors suggested that the difference in the results could be due to different agronomic conditions.
   2. The Loiseau et al., (2017a) study conducted new tests, using culture conditions similar to those described by Bertolini et al. (2015): this made it possible to confirm the results obtained during their first experiment.
   3. The Oishi et al. (2017) did not identify a seedling contaminated with Clso and do not confirm transmission of CLso from seed to plant.
   4. According to the study Haapalainen et al. (2018), Lso was not detected in carrot crops grown from seed lots known to be infected with the D Lso haplotype. This result is consistent with the results of the studies by Loiseau et al. (2017a, 2017b).
   5. The Mawassi et al. (2018) also did not observe seed transmission of the disease. It suggests that psyllids are the main vectors of the disease.
   6. The Carminati et al. (2019) also did not observe CLso transmission by semen during the tests carried out.
   7. The tests from the Fujikawa, T. et al. (2020) used infected seed lots from France and were carried out under the same conditions as the Bertolini and Loiseau studies. The results confirmed that the rate of transmission of the bacteria is insignificant.
2. Regarding **the results** of Bertolini et al. (2015):
   1. After 6 months of cultivation, the rate of infected plants found was heterogeneous according to the batches (15 to 42%), but the results of other studies showed:
      * In the Loiseau et al. Study (2017b), out of 432 plants, only 9 plants were detected positive for CLso (i.e., 2% of cases), while the infection rate of the seed lots was 96% to 100 %, higher than the infection rate of the batches used in the Bertolini study (47 to 94%). Hence, the transmission of the bacteria is therefore considered rare and difficult to reproduce.
      * In the Fujikawa study, only 2-6% of the cases tested positive: a very low proportion. In particular, this study made it possible to statistically assess the risk of infection represented by CLso. It was concluded that transmission by seeds is highly insignificant.
      * In the Oishi and Carminati studies, no positive cases were detected.
      * In Mawassi's study, no positive case was detected even though, 30% of the seed lots were initially infected.
   2. In addition, in the Bertolini study, plants positive for *CLso* did **not show visible symptoms** (except for a specific case representing less than 0.7% of the sample).
      * In the studies Loiseau et al., (2017a & b) and Fujikawa, T. et al. (2020), no symptoms were detected, even in cases that tested positive.
   3. In addition, field trials were carried out in the Bertolini study and observed that after 6 months, 100% of the plot was infected with the bacteria. This result should be **interpreted with great care**, because no record, no analysis was provided, neither details of the experimental protocol or the cultivation conditions. Having so little information, it is not possible to conclude that the seeds are the factor in the infection of the plot, this may also be due to other vectors, such as psyllids.
3. Candidatus Liberibacter solanacearum is identified as a **vascular bacterium limited to the phloem** :
   1. The bacteria cannot be cultivated in an artificial medium and can only survive in the phloem of the plant or an insect vector (Bové, 2006; Jagoueix et al., 1994; Liefting et al., 2009b). In the case of seeds, the bacteria would have been localized in the phloem of the seed coats (Bertolini et al., 2015; Mawassi et al., 2018; Haapalainen, M. et al., 2018)
   2. According to the Loiseau et al. (2017b), several reports, on pests limited to phloem / xylem, concluded that transmission of this type of pathogen is very rare and difficult to reproduce (Della Coletta-Filho et al., 2014; Hartung et al., 2014; Lapierre & Signoret, 2004).
   3. According to the Haapalainen study, M. et al. (2018), the bacteria detected in the seeds may be non-viable or unable to pass from seed coats to the embryo: the suspensor connecting the embryo to the funicle does not contain phloem (Standler et al., 2005). Therefore, CLso would be limited to phloem cells (Nissine et al., 2014).
   4. These data therefore provide evidence that transmission of the bacteria by seed is unlikely.
4. Studies conclude that **CLso is transmitted by a vector other** than **seeds: Psyllids** :
   1. According to several studies (Munyaneza, 2012; Munyaneza, 2016; Haapalainen et al., 2017; Nissinen et al., 2014) and EPPO, the bacteria is known to be transmitted by psyllids.
   2. Psyllids have been observed to be particularly abundant in areas where CLso is known, making them the main vector of the pathogen (EPPO; Mawassi et al., 2018).
   3. When psyllids feed on the phloem contents, they introduce Lso bacteria into the phloem tissue of the seed coat (MPI Pest Risk Assessment, 2015; Monger and Jeffries, 2016). Seed-borne pathogens must be detected from the seed embryo (Singh and Mathur, 2004)
5. The **geographic distribution of CLSo haplotypes** around the world suggests that seeds do not transmit the bacteria in importing countries.
   1. Indeed, the three known haplotypes infecting carrots (C, D and E) are localized as such: haplotype C in Northern Europe (Sweden, Norway, Finland, and Germany) and transmitted by T. apicalis; haplotypes D and E in Southern Europe / Mediterranean (Spain, France, Canary Islands, Africa and Israel) transmitted by B. trigonica (Alfaro-Fernandez et al.2012a, b; Hajri et al.2017; Munyaneza et al.2010, 2012, 2015; Tahzima et al.2014).
   2. In addition, recent work has demonstrated the presence of the bacteria in European seed lots since the 1970s (Monger and Jeffries, 2018). Due to the intensity of international trade, the bacteria should have spread globally. However, it can be assumed that the presence of CLso has not yet been identified in importing countries.
   3. The work of Haapalainen et al. (2018) in Finland identified haplotype D on 2 of 34 imported carrot seed lots. This haplotype has never been observed in Finland’s’ cultivation, where only haplotype C has been reported. Since carrot seeds are not produced in Finland, they are exclusively imported: thus, it would have been likely to identify the other haplotypes of CLso in Finnish crops, but this has never been the case. Thus, as the authors of this publication concluded, this suggests that carrot seeds are not a major source of transmission of Lso.
   4. The work of Mawassi et al. (2018) identified the presence of haplotype D in carrot crops in Israel. The origin of CLso in this country is still unknown. Several scenarios were considered in this study: CLso could be the result of imported plant materials, the migration of psyllids or the bacteria could have been present in Israel for a long time. Tests were then carried out on imported seed lots from 20 years ago: these revealed the presence of a haplotype similar to haplotype E. Israel imports carrot seed from countries known to have CLso; thus, it would be likely that haplotypes C, D and E are present in Israeli crops. However, haplotypes C and E have so far not been detected in carrot crops in Israel.
6. To conclude, the results of the Bertolini et al. (2015) were disputed by the seven studies presented. Thus, the study alone does not represent sufficient evidence to clearly demonstrate transmission of CLso by seeds.
   1. The positive results obtained by Bertolini et al. (2015) are most likely due to cross contamination during DNA extraction or PCR amplification and/or non-specific amplification of organisms closely related to Liberibacter.

# Other reasons

1. Seed transmission has not been clearly demonstrated for other Liberibacter species (Hilf et al. 2013, Hilf 2011, Van Vuuren et al. 2011).*”*
   1. In potatoes, this bacterium is not transmitted by true seeds (Munyaneza, 2012).
   2. Likewise, in the first studies on transmission by citrus seeds of “Candidatus Liberibacter asiaticus”, the possibility of vertical transmission of this bacterium was debated (Tatineni et al. 2008; Tirtawidjaja 1981). However, intensive research by Hartung et al. (2010) have never found typical symptoms of Huanglongbing (yellow dragon disease, produced by Candidatus Liberibacter spp.) Nor positive tests for 'Ca. L. asiaticus” during three years of trials on 723 plants grown from seeds collected from infected citrus fruits. Currently, there is still no evidence of its transmission through citrus seeds. Previous reports of seed transmission of the pathogen have never been confirmed.
   3. As part of internal work carried out by ANSES, plants germinated from seeds of infected tomato, pepper and tamarillo fruits were all tested negative for *CLso*.
2. As a reminder, only the CLso bacteria of Solanaceae haplotypes (A and B), as well as its vector *Bactericera cockerelli*, are included in the A1 list of EPPO: "List of pests recommended for regulation as quarantine pests" (according to the most recent version of September 2020). Apiaceae haplotypes (C, D, E) are not considered to be potential quarantine pests.

# Conclusion

These recent studies confirm that **the transmission** of the bacterium Candidatus Liberibacter Solanacearum by seeds of carrots and Apiaceae **has not been demonstrated**. Thus, the measures established by Turkey for imports of carrot seeds (negative PCR test required) are no longer technically viable.

Based on these scientific sources, **Japan**, **New Zealand**, two particularly restrictive countries on phytosanitary standards, as well as **Costa Rica** and Argentinahave withdrawn their requirements on Candidatus Liberibacter Solanacearum, in 2020 and 2021.

We therefore ask the Peruvian Health Authorities to withdraw the PCR analysis requirements of CLso on carrot seeds so that French imports can once again be operational.