# Search for reservoirs of '*Candidatus* Liberibacter solanacearum' and mollicutes in weeds associated with carrot and celery crops

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**Abstract**

Currently, the main arthropod vectored pathogens associated with carrot and celery crop diseases are `*Candidatus* Liberibacter solanacearum´, *Spiroplasma citri* and different phytoplasmas species. Mitigation strategies require elucidating whether these pathogens survive in the weeds of these *Apiaceae* crops, which can act as reservoirs. Weed surveys were conducted in a vegetative cycle (April to October 2012) in the spontaneous vegetation that surrounded crops affected by `*Ca.* L. solanacearum´, *S. citri* and/or phytoplasmas. Sixty-three species of 53 genera that belong to 23 botanical families were collected in the main carrot and celery Spanish production area. Species were identified, estimating coverage and abundance, and conserved in herbarium. Samples were analysed by nested-PCR with universal primers for phytoplasmas detection, and were sequenced for identification purposes; by conventional PCR for *S. citri* and real-time PCR for `*Ca.* L. solanacearum´. The only detected pathogens were `*Ca.* Phytoplasma trifolii´ (clover proliferation group 16Sr VI-A) in *Amaranthus blitoides* and *Setaria adhaerens* and `*Ca.* P. solani´ (stolbur group 16Sr XII-A) in *Convolvulus arvensis*. These pathogens were also sporadically detected in celery or carrot crops. Unexpectedly, neither `*Ca.* L. solanacearum´ nor *S. citri* was detected in the weed samples, despite the relatively high prevalence of these pathogens (less than 66% and 25%, respectively) in the surveyed plots. This suggests that weeds do not play an epidemiological role as reservoirs in the spread of such organisms in the studied region. The use of pathogen-free seed lots and the control of vectors are crucial for preventing the introduction and spread of these economical important pathogens to new areas.

**Keywords:** spontaneous vegetation, *Spiroplasma citri*, phytoplasmas, PCR, detection.

Celery (*Apium graveolens* L.) and carrot (*Daucus carota* L.) are long-season crops of increasing interest in European countries. In Spain, approximately 1,600 and 7,300 ha of celery and carrot are respectively cultivated in three overlapping cycles (early, medium and late) from March to December. These *Apiaceae* crops are affected by several fungal, viral and bacterial pathogens, which can cause major crop loss (Davis and Raid 2002). Currently the main pathogens associated with *Apiaceae* crops in Spain are `*Candidatus* Liberibacter solanacearum´, *Spiroplasma citri* and phytoplasmas, which are associated with vegetative disorders and yellowing (Cebrián et al. 2010; Alfaro-Fernández et al. 2012; Teresani et al. 2014). `*Ca.* L. solanacearum´ is a carrot seed-borne pathogen that also horizontally spreads by several psyllid species in a persistent manner (Bertolini et al. 2015; Haapalainen 2014). *S. citri* and phytoplasmas are persistently spread by *Cicadellidae* species, and phytoplasmas also by *Fulgoridae* and *Psyllidae* (Bové 1986, Bertaccini and Duduk 2009). This study was undertaken to: (i) identify the weed species that grow in celery and carrot crop fields and surrounding areas; (ii) determine the prevalence of `*Ca.* L. solanacearum´, *S. citri* and phytoplasmas in weed species; and (iii) assess the putative role of weed species as reservoirs in order to design mitigation strategies.

To estimate the prevalence of pathogens in carrot and celery crops in the weed-surveyed area, sampling was performed in six celery and three carrot plots. Groups of 10 contiguous plants were sampled in 10 areas in each plot. These 100 plants were analysed at the end of each (early, middle and late) cultivation cycle for the three pathogens. Weed surveys were conducted in the spontaneous vegetation inside and in the surrounding area of the celery and carrot crops (six different areas) located at Villena (Alicante, east Spain) on four different dates in 2012 (April, May, July and October). Weeds were sampled in areas of 100 $m^2$ placed on the margins of cultivation plots. They were identified *in situ* whenever possible, or were codified and identified using different botanic weed guides (Carretero 2004; Mateo and Crespo 2009) and online herbariums (Flora Ibérica 2012; Flora Vascular 2012; Herbario de la Universidad Pública de Navarra 2012; Herbario Virtual del Mediterráneo Occidental 2012; Weed Science Society of America 2012). The identified specimens were conserved in the IAM-UPV herbarium. A botanical inventory was made following Braun-Blanquet's (1932) methodology, and using the cover and abundance scales of Fujiwara (1987). Leaf samples of the various weed species, including vascular tissues, were collected, placed into plastic bags and stored at 4ºC until analysed.

Total DNA was extracted from the plant material using cetyltrimethylammonium bromide (CTAB) buffer and the DNeasy Plant Mini Kit (Qiagen, USA), as described by Green et al. (1999). Samples were analysed for `*Ca.* L. solanacearum´ detection by real-time PCR according to Teresani et al. (2014) (Calsol/100, Plant Print Diagnostics kit, Spain), and also for *S. citri* by conventional PCR with primers p89-F/p89-R described by Yokomi et al. (2008). Nested-PCR was carried out using universal phytoplasma primers P1/P7 (Schneider et al. 1995) in the first amplification, followed by R16F2n/R16R2 (Gundersen and Lee 1996) in the second amplification. The total DNA from the reference phytoplasmas of the different groups were utilised as positive controls: 16SrI-A (tomato big bud, BB), 16SrI-B (aster yellows, AY), 16SrII (peanut witches´ broom, PnWB), 16SrIII-A (x-disease, CX), 16SVI-A (potato witches´ broom, PWB), 16SrX-A (apple proliferation, AP) and 16SrXII-A (stolbur, STOL). PCR products were analysed in 1.2% agarose gels, stained in ethidium bromide and visualised with a UV transilluminator. The fragments amplified by nested-PCR were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Germany) and directly sequenced to confirm the identity of the detected phytoplasmas species.

Sixty-three species of 53 genera that belong to 23 botanical families were collected. The identification and relative abundance and coverage of the weed species collected in the celery and carrot fields and surroundings are detailed in Table 1. Vegetation species basically belonged to the families *Amaranthaceae*, *Brassicaceae*, *Compositae*, *Convolvulaceae*, *Cyperaceae*, *Poaceae*, and *Urticaceae*. Of the total, 21 weed species presented a cover percentage above 5% (cover level > 2) in at least one survey. This cover level was considered the reference level to estimate the species with higher relative abundance. Depending on the season during which the survey was conducted, the species of weeds present in the plots differed; e.g. in April and May, *Brassicaceae* species were more relevant given their adaptation to the cold season, whereas species *Convolvulus arvensis* L. and *Solanum nigrum* L. were prevalent in October as they flowered in summer or early autumn. It is noteworthy that *Salsola kali* L. has been reported as reservoir for the Mediterranean vector *Circulifer haematoceps* of *S. citri* (Bové et al. 1988), and that *S. vermiculata* L. were highly abundant in two different areas.

In celery crops, none of the tested pathogens (`*Ca.* L. solanacearum´, *S. citri* and phytoplasmas) was detected at the end of the early cultivation cycle (June), whereas only

*S. citri* was detected in the middle one (August) with a prevalence that ranged from 5% to 25%, and the prevalences of `*Ca.* L. solanacearum´ and phytoplasmas were detected in the late cultivation cycle (November), and ranged from 0-6% to 0-4%, respectively. The range of prevalence corresponded to the minimum and maximum percentage of the infected plants detected in the different plots surveyed throughout the vegetation cycle. The identified phytoplasma species was `*Ca.* P. trifolii´ (subgroup 16Sr-VI-A, accession numbers KP099581- KP099582). In the carrot crops, `*Ca.* L. solanacearum´ was detected with a 4% prevalence at the end of the early cultivation cycle (June), and with 66% for the middle one (January) and 18% for the late one (November). *S. citri* was not detected, but occasionally in the late cultivation cycle, phytoplasmas were detected with a 2% prevalence. The detected species was identified as `*Ca.* P. solani´ (subgroup 16Sr-XII-A, accession numbers KP099588-KP099586). The prevalence of infection in the carrot and celery crops obtained in this study was similar to those reported in previous works in 2012, but was lower than that observed in previous years in the same area (Teresani et al. 2014).

Phytoplasma infection was confirmed in three weed species, namely *A. blitoides*, *S. adhaerens* (collected in July) and *C. arvensis* (collected in October). The BLAST analyses revealed that the sequences of the phytoplasmas detected from *A. blitoides* (Accession Number KP099583) and *S. adhaerens* (Accession Number KP099584) shared more than 99% nucleotide identity, and had different phytoplasma strains which belonged to `*Ca.* P. trifolii´ (clover proliferation group 16SrVI-A; accession number EU649681). However, the phytoplasma detected in *C. arvensis* was identified with 99% nucleotide sequence identity with `*Ca.* P. solani´ (stolbur group 16SrXII-A; accession number KP099587). *C. arvensis* is a perennial weed, whereas *S. adhaerens* and *A. blitoides* are annual, although their biological cycle is not relevant in an area where the Apiaceae crops are intensively cultivated along the year with three different overlapping cycles. Both phytoplasmas species presented a wide range of hosts. `*Ca.* P. trifolii´ has been reported to infect several species, such as clover (Lee et al. 1998), strawberry (Jomantiene et al. 1999), onion (Lee et al. 2001), bean (Lee et al. 2004) and hazelnut (Jomantiene et al. 2000), among others. This work is the first to report this phytoplasma species in celery, and even in *A. blitoides* and *S. adhaerens*, but the wide host range of this pathogen indicates that these plants and others present in that area are potential hosts. So a thorough control should be undertaken to prevent spread, even more when those weed species are

present at a high density as showed the cover/abundance scored. `*Ca.* P. solani´ has already been detected on *C. arvensis* in other European countries (Fialová et al. 2009; Ember et al. 2011).

Unexpectedly, neither `*Ca.* L. solanacearum´ nor *S. citri* was detected in the surveyed weed species, although *Solanum dulcamara* L. has been reported as a natural host of `*Ca.* L. solanacearum´ (Murphy et al. 2014), and several species collected in this survey were reported as natural non-rutaceous hosts of *S. citri* as *Echium* sp., *Sysimbrium irio* L. or *Convolvulus arvensis* L. (Nejat et al. 2011). Therefore, weeds do not act as a reservoir, at least not in an area where these pathogens have been historically associated with major economic loss in celery and carrot crops during 2012. However, the year of this survey was atypical because the prevalence of arthropods was low compared with the previous year and the rainfall was twice than that in 2011. From the arthropods caught, Cicadellidae was the most common family (60.2%) compared with Aphididae (31.5%) and Psylloidea (8.2%), although *Ca.* L. solanacearum was detected in *Bactericera trigonica* and *B. tremblanyi* which presented their maximum population peaks in the early cycle (April and July) and in the late cycle (August), respectively (Teresani et al. 2015). In this survey, the three pathogens were detected in the crop species, but only phytoplasmas were present in weed species. Apparently, weeds do not play an epidemiological role in the spread of these organisms in the surveyed area. Consequently, the use of pathogen-free seed lots and the control of the psyllid and leafhoppers have to be considered relevant measures to prevent these economical important pathogens from being introduced into and spreading to new areas.

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