Australasian Plant Disease Notes (2018) 13: 13
https://doi.org/10.1007/s13314-018-0298-1

Cota tinctoria and *Orosius albicinctus*: A new plant host and potential insect vector of '*Candidatus* Phytoplasma trifolii'

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Received: 3 March 2018 / Accepted: 26 April 2018 / Published online: 2 May 2018

Abstract
Samples of *Cota tinctoria* with phytoplasma symptoms including witches' broom, stunting, twisting of the shoots and little leaf were observed in Bandar Abbas, Iran, in 2017. Phylogenetic analysis revealed that the phytoplasma associated with *Cota tinctoria* witches' broom (CtWB) is a strain of '*Candidatus* Phytoplasma trifolii'. Furthermore, the phytoplasma was successfully detected in the leafhopper, *Orosius albicinctus* collected from the symptom-bearing plants. This work therefore reports a new phytoplasma-associated disease on *Cota tinctoria* and its putative insect vector.

Keywords Golden marguerite · Phytoplasma · Potential vector · Witches' broom

Phytoplasmas (wall-less and phloem-restricted plant pathogenic bacteria) are recognized as a serious constraint for the cultivation of many ornamental plants around the world. They can reduce the quality and yield of ornamental plants because of their unspecific symptoms and can cause severe losses (Bertaccini and Duduk 2010). Epidemics of these diseases have limited the cultivation of many ornamental plant species such as gladiolus, lily, chrysanthemum and rose. Phytoplasmas affect more than 600 plant species from tropical, subtropical to temperate climates all over the world (Jones 2002). The general symptoms include flower malformation, growth abnormalities, yellowing of leaves, elongation and etiolation of internodes, witches' broom, stunting, little leaf and virescence (Chaturvedi et al. 2010). In plant disease epidemics, insect vectors play a major role. Furthermore, polyphagous vectors have the potential to inoculate a wide range of plant species, which may differ in susceptibility (Weintraub and Beanland 2006).

Cota tinctoria, also known as golden marguerite, yellow chamomile, is a species of perennial flowering plant in the Asteraceae family. It is a short-living plant often treated as biennial, native to Europe, the Mediterranean and Western Asia and naturalized in scattered locations in North America (Franke 2005). It produces excellent yellow, buff and golden-orange dyes, used in the past for fabrics. This species is grown in gardens for its bright attractive flowers and fine lacy foliage, and in Iran, especially in southern regions, this species is widely planted as an ornamental plant in green landscapes. So far, no diseases and pests have been reported on *Cota tinctoria*.

In January 2017, during a survey, typical symptoms of phytoplasma disease, including witches' broom, stunting, twisting of the shoots and little leaf were observed in several *Cota tinctoria* flowers planted in a green landscape in Bandar Abbas, Hormozgan province, Iran (Fig. 1). A preliminary study to determine whether a phytoplasma was associated with the symptom-bearing plant was performed. Simultaneously, insect samples, mainly planthoppers and leafhoppers (Cicadellidae), were collected using yellow sticky cards. The yellow sticky cards were replaced at weekly intervals and species were captured and checked for phytoplasma presence. Insects were preserved in absolute acetone until DNA extraction (Fukatsu 1999).

Ten samples of both symptom-bearing and three samples of asymptomatic *Cota tinctoria* were collected from green landscapes of Bandar Abbas (N27°22′36″; E56°35′25″). Total DNA was extracted from leaves of all collected symptom-bearing and symptomless samples by using the cetyltrimethylammoniumbromide (CTAB) extraction procedure described by Sahu et al. (2012). Total DNA was extracted from ten individual leafhoppers using the CTAB protocol described by Reineke et al. (1998).

To determine the presence of phytoplasmas in *Cota tinctoria* and insect samples, a nested polymerase chain

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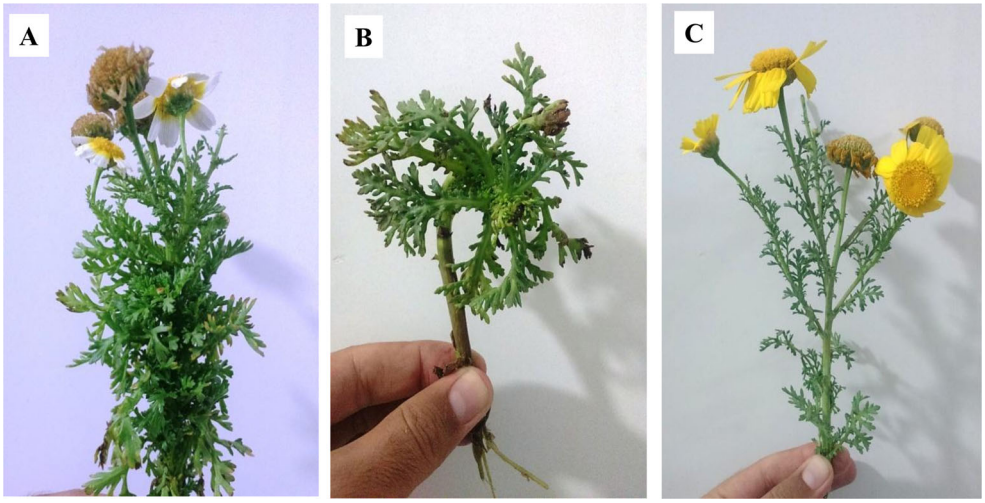

Fig. 1 Symptoms of witches' broom, little leaf (**a**), stunting and twisting of the shoot (**b**) in comparison with healthy *Cota tinctoria* (**c**)

reaction (PCR) was employed using the universal primers P1/P7 (Deng and Hiruki 1991) followed by R16F2n/R16R2 which amplifiy a phytoplasma16S rRNA fragment (approximately 1.25 kbp). PCR assays were performed with 50 μl reactions containing 1 μM of each primer, 25 μl master mix (Ampliqon, cat. no. A190303, Denmark), 20 μl dd H_2O and 3 μl template DNA. PCR conditions for amplification were denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 55 °C for 1 min and 72 °C for 1.5 min, with a final extension of 10 min at 72 °C. One microlitre of 1:10 diluted first amplification product was used as DNA template in the second round (nested) PCR. Nested-PCR conditions were the same used in a first round, while annealing temperature for the second round was 56 °C. *'Candidatus* Phytoplasma aurantifolia' and DNA template free were used as positive and negative control, respectively. To identify the insect species, PCR amplification of partial mitochondrial COI (cytochrome oxidase I) gene was performed using LCO1490 and HCO2198 primers (Folmer et al. 1994). The PCR was performed in 25 μl solution contained 12.5 μl master mix (Ampliqon, cat. no. A190303, Denmark), 1 μl of each primer (10 pmol/ μl), 1 μl of extracted DNA and 9.5 μl dd water. The thermocycling program consisted of an initial denaturation step at 95 °C for 3 min, followed by 5 cycles of 1 min at 94 °C, 1 min at 45 °C, 1 min at 72 °C, and then 35 cycles of 1 min at 94 °C, 1 min at 51 °C, 1 min at 72 °C, with a final extension step at 72 °C for 5 min.

The amplified fragments were electrophoresed on 1.0% agarose gel stained with FluoroDye under UV light and phytoplasma was detected as nested PCR products of approximately 1250 bp (16S rRNA) from five separate symptomatic plants using the R16F2n/ R16R2 primer pair. Similarly, amplification products were obtained from positive control samples but not in the assays of samples from non-symptomatic plants and negative controls. Insects collected on the symptom-bearing plants were also identified and checked for phytoplasma presence using PCR assays and *Orosius albicinctus* (Distant, 1918) was the dominant and only positive species in which PCR and subsequently sequencing results confirmed presence of the phytoplasma. Eight out of ten tested individuals showed PCR bands of the correct size, confirming presence of phytoplasma.

To confirm the presence of phytoplasma and for phylogenetic analysis, two fragments (one plant, one insect) were randomly selected from the nested PCR round and sequenced bidirectional by using primer R16F2n/ R16R2 while one fragment of COI gene was sequenced directly using LCO1490 and HCO2198 primers. The obtained COI sequence was analysed using GenBank, by NCBI-BLAST to find the closest match. Raw sequence chromatograms were assembled and edited using DNAstar (Hall 1999) to correct ambiguous bases or remove low quality stretches from termini of the sequences. Homologies to known sequences were detected using the BLASTN algorithms against the non-redundant GenBank database. Phylogenetic analyses were conducted by neighbor joining (NJ) methods using MEGA 6.0 software (Tamura et al. 2013). The 16S rRNA sequences of phytoplasma used in a comprehensive phylogenetic analysis were downloaded from GenBank (the accession numbers are given in brackets in fig. 2).

BLAST analysis of the partial 16S rRNA sequence from the plant sample (Accession No. MG569790) revealed that the phytoplasma associated with *Cota tinctoria* witches' broom shared 99% identity with some phytoplasmas related to the 16SrVI phylogenetic group *'Candidatus* Phytoplasma trifolii' such as Tomato big bud (accession no. JF508508), '*Candidatus* Phytoplasma trifolii' (KX773529), Zuccini phyllody (accessionno. KP119494). The phylogenetic tree was in accordance with BLAST analysis and the sequence from the present study was clustered in group 16SrVI (Fig. 2). This result was further confirmed by the analysis using the *i*PhyClassifier online tool (Zhao et al. 2009) (http://

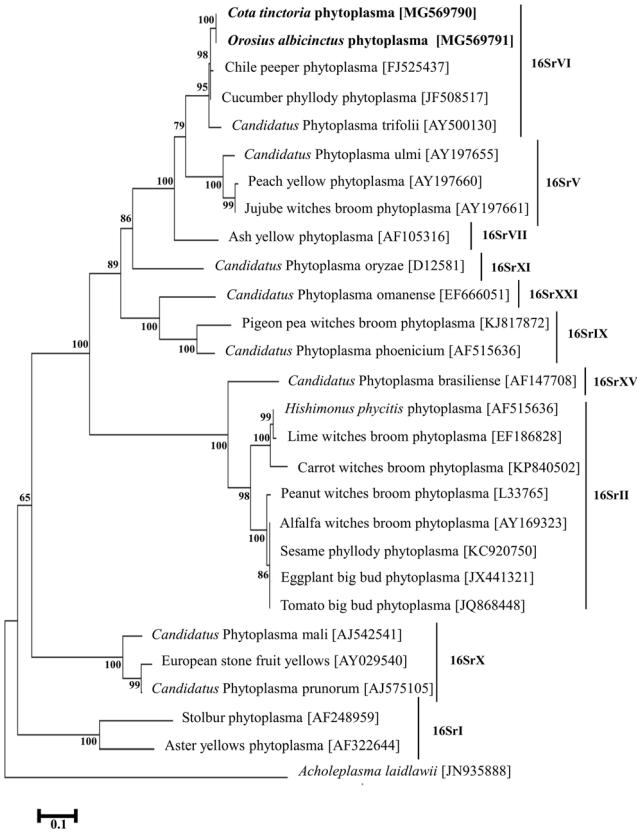

Fig. 2 Phylogenetic tree of partial 16S rRNA gene sequences from *Cota tinctoria* witches' broom phytoplasma isolates (marked in bold) and selected phytoplasma reference sequences. GenBank accession numbers are shown in brackets, and 16Sr groups are annotated to the right. *Acholeplasma laidlawii* was used as the outgroup to root the tree. The tree was constructed by the neighbor-joining method using MEGA 6 software. The bar indicates the number of nucleotides substitution per site. Bootstrap values are shown at nodes with greater than 50% support

plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) where it was determined that the *C. tinctoria* phytoplasma was related to 16SrVI group, subgroup A.

The sequence obtained from insects was aligned (Clustal Omega) with that obtained from symptomatic plants which revealed 100% similarity. The nucleotide sequence of the phytoplasma isolated from insect was deposited in the GenBank database (Accession No. MG569791) and the sequence obtained from the insect was placed in a clade with that isolated from plants (Fig. 2). Insect COI gene sequence showed 100% identity with *Orosius albicinctus*. Based on these results, *O. albicinctus* can be considered as a potential vector of this disease on *C. tinctoria* in this region in southern Iran. This species has been reported previously as a vector of alfalfa witches' broom, cucumber phyllody, garden beet witches' broom and sesame phyllody (Omidi et al. 2010) in Iran which reinforces the hypothesis that this species could be an insect vector of this disease to other flowers. However, vectoring capability can only be confirmed by transmission assays. *Orosius albicinctus* is distributed in Iran, Australia, Fiji, Indonesia, New Britain and Gava, Israel, Egypt, India (Pakarpour Rayeni and Seraj 2016).

Based on the virtual RFLP results and phylogenetic analysis of the 16S rRNA gene sequences, it can be concluded that the phytoplasma detected in the symptomatic *C. tinctoria* in Hormozgan province belongs to the 16SrVI group, subgroup A. In Iran, group16SrVI phytoplasmas have been identified in a number of hosts including *Carthamus tinctorius*, *Cucumis sativus*, *Medicago sativa* and *Suaeda aegyptiacae* (Askari Seyahooei et al. 2017). To the author's knowledge, this is the first report of a phytoplasma disease associated with *Cota tinctoria* plants with a substantial population of the *O. albicinctus* leafhopper as a potential vector.

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