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RAPID COMMUNICATION

First report of phytoplasma detection on sand olive, cowpea and alfalfa in Iraq

Nawres Al-Kuwaiti[1]*, Tareq Kareem[1], Feryal H. Sadaq[1], Laith H. AL-Aadhami[2]

[1] Plant Protection Department, College of Agricultural Engineering Sciences, University of Baghdad, Iraq

[2] Parks and Afforestation Directorate, Mayoralty of Baghdad, Al-Adhamiyah, Baghdad, Iraq

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*Corresponding address:
N.Alkuwaiti@coagri.uobaghdad.edu.iq

Abstract

The association of phytoplasma was investigated in sand olive [*Dodonaea viscosa* ssp. *Angustifolia* (L. f.) J.G. West], cowpea [*Vigna unguiclata* (L.)] Wap and alfalfa (*Medicago sativa* L.) plants exhibiting witches broom, fasciation and little leaf symptoms, respectively. Sequence analysis of ~1.7 kb DNA fragments amplified by P1/P7 primer set confirmed the association of '*Candidatus* Phytoplasma aurantifolia' within symptomatic alfalfa, while '*Ca*. Phytoplasma cynodontis' was associated within cowpea and sand olive.

Keywords: 16SrII-D phytoplasma, 16SrXIV-A phytoplasma, flat stem, *in silico* RFLP, white leaf

Phytoplasmas are some of the most devastating plant pathogens that impact a wide range of crops worldwide causing serious losses (Bertaccini *et al*. 2014). In Iraq, phytoplasma diseases have been reported since 1998, however the detection of the pathogen was not confirmed until recently based on molecular approaches (Al-Kuwaiti *et al*. 2015). In a previous study, two phytoplasmas belonging to subgroups 16SrXIV-A and 16SrII-D were characterized in Iraq based on 16S rDNA sequences (Al-Kuwaiti *et al*. 2017). They were associated with white leaf and witches broom diseases on Bermuda grass and tomato and eggplant, respectively. In addition to witches broom and yellowing, the association of '*Candidatus* Phytoplasma australasia' with little leaf symptoms in alfalfa was reported in Iran (Hosseini *et al*. 2013), however, the species was invalidated by the IRPCM and associated strains were reassigned to '*Ca*. Phytoplasma aurantifolia' (IRPCM 2004). Despite the association with white leaf disease in several hosts in India (Rao *et al*. 2017b), '*Ca*. Phytoplasma cynodontis' was reported to be associated with witches broom and fasciation (flat stem) symptoms in sand olive in Saudi Arabia (Omar 2016) and cowpea in India (Rao *et al*. 2017a).

Sand olive, (*Dodonaea viscosa*), a member of the family Sapindaceae is a small, evergreen shrub about 7 m in height (Hossain 2018). Sand olive is thought to originally come from Australia, however now it is widely distributed in different geographical regions (Gilman 1999; Al-Snafi 2017). In Iraq, *D. viscosa* is grown and propagated as an ornamental plant used for decoration, shading and hedging, due to its rapid growth rate and high tolerance to drought (Gilman 1999). Also, chemical analysis showed that *D. viscosa* has chemical compounds which could potentially be used for medicinal, insecticidal, and pharmaceutical applications (Hamadi 2017).

Cowpea, *Vigna unguiclata* (L.) Walp, and alfalfa, *Medicago sativa* L. are leguminous crops within the family Fabaceae. Cowpea is grown and consumed worldwide for its high protein and carbohydrate content (Jayathilake *et al*. 2018). Both cowpea and alfalfa can be used as forage and cover crops due to their ability to fix nitrogen, improve poor soils and prevent erosion (Clark 2012). In Iraq, the estimated cowpea production of dry seeds was 68 tons (FAO 2017), whereas alfalfa production scored 82,2812 tons (CSO 2018). Alfalfa is grown for a duration of 2–4 years, while cowpea

is cultivated only during the summer season, as an alternative to other vegetable crops during drought seasons in Iraq (Al-Kuwaiti 2013). Recently, phytoplasma like symptoms were observed in sand olive, alfalfa and cowpea growing areas near Baghdad. Using a simplified molecular technique for phytoplasma detection (Liu *et al.* 2017), this study was aimed to confirm phytoplasma association with diseased sand olive, alfalfa and cowpea plants using molecular approaches.

Leaf samples from sand olive (n = 2), alfalfa (n = 5) and cowpea (n = 2) exhibiting phytoplasma disease symptoms (Fig. 1 A–C) were collected from the Al-Jadriya region (33° 16' 9.336" N; 44° 21' 44.46" E) near Baghdad. Total DNA was extracted from each sample and P1/P7 based polymerase chain reaction (PCR) (Deng and Hiruki 1991; Smart *et al.* 1996) was performed according to Al-Kuwaiti *et al.* (2017). PCR products were analyzed using a gel electrophoresis approach described by Sambrook and Russell (2006) with a slight modification using GreenStar™ Nucleic Acid Staining Solution I (Bioneer, South Korea) instead of Ethidium Bromide (EtBr) following the manufacturer's instructions. PCR products including ~1.7 Kb DNA fragments were sent to Macrogen Inc., South Korea for sequencing. The obtained sequences were analyzed and compared to equivalent GenBank sequences performing BLAST comparison using MEGA X software (Kumar *et al.* 2018). Virtual restriction fragment length polymorphism (RFLP) was generated to confirm phytoplasma group/subgroup identification using *i*PhyClassifier (Zhao *et al.* 2009). GenBank accession numbers (MK367411-MK367419) were assigned to phytoplasma sequences which were obtained.

Sequence comparison confirmed the detection of phytoplasma when all ~ 1.7 Kb DNA fragments amplified from the symptomatic samples showed high identity percentages to 16S rRNA phytoplasma genomic region (Deng and Hiruki 1991; Smart *et al.* 1996). Sequences obtained from sand olive and cowpea shared 99.88% maximum nucleotide (nt) identity with '*Ca.* Phytoplasma cynodontis' from Albania (KF383980). All five sequences from alfalfa exhibiting little leaf symptoms scored 97–99% maximum nt. identity to '*Ca.* Phytoplasma aurantifolia' associated with faba bean phyllody disease in Iran (KP869129). The *in silico* analyses using *i*PhyClassifier confirmed the group/subgroup assignment of the phytoplasma associated with sand olive witches broom, cowpea flat stem and alfalfa little leaf diseases. The virtual RFLP patterns derived from Sand olive-Sand olive1, Cowpea-Cowpea1 and Alfalfa2-Alfalfa4 16S rDNA F2nR2 fragments were identical (similarity coefficient 1.00) to the reference patterns of the 16Sr group XIV- A (GenBank accession: AJ550984) for sand olive and cowpea and 16Sr group II-D (GenBank accession: Y10097) for alfalfa samples, respectively (Zhao *et al.* 2009). Alfalfa 1 was a variant of subgroup 16SrII-D and shared 0.98 similarity coefficient with the reference strain. Phylogenetic analysis resulted in two distinct clusters representing phytoplasma subgroups 16SrII-D and 16SrXIV-A, supporting these findings (Fig. 2). According to IRPCM, this strain was reassigned to '*Ca.* Phytoplasma aurantifolia' (IRPCM 2004). Data from this study confirmed the association of this phytoplasma with symptomatic alfalfa samples collected near Baghdad. In a recent study, this phytoplasma was reported to impact other commercial crops (i.e. tomato and eggplant) in the Basra province (Al-Kuwaiti *et al.* 2017). Alfalfa may act as an alternative host and a potential source of '*Ca.* Phytoplasma aurantifolia' for its vectors. The perennial life cycle (FAO 2019) and mild symptoms enable alfalfa to survive longer in growing fields. Most farmers follow a mixed culture system in Iraq and due to water shortage and soil salinity problems in Iraq, alfalfa is cultivated intensively with other vegetables throughout the country (CSO 2018). '*Ca.* Phytoplasma cynodontis' associated with Bermuda grass white leaf disease was reported in Baghdad province (Al-Kuwaiti *et al.* 2017). In this study, it was shown that '*Ca.* Phytoplasma cynodontis' was associated with

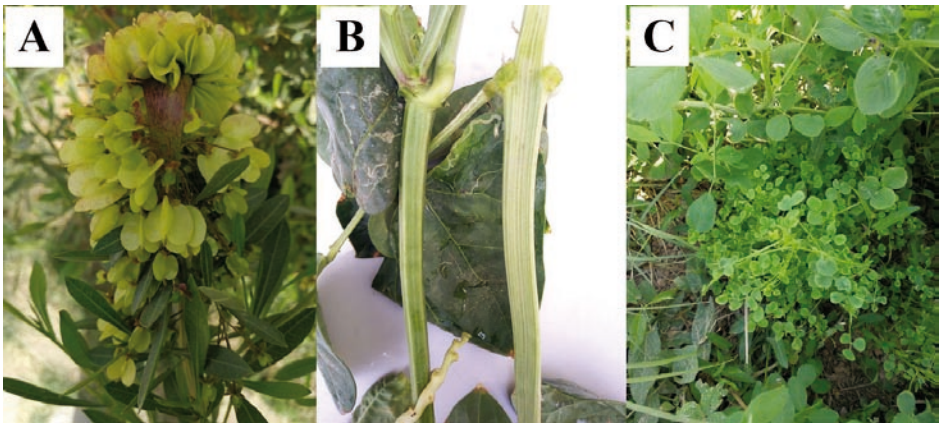

Fig. 1. A – symptomatic plants exhibiting witches broom on sand olive, B – flat stem on cowpea, C – little leaf on alfalfa

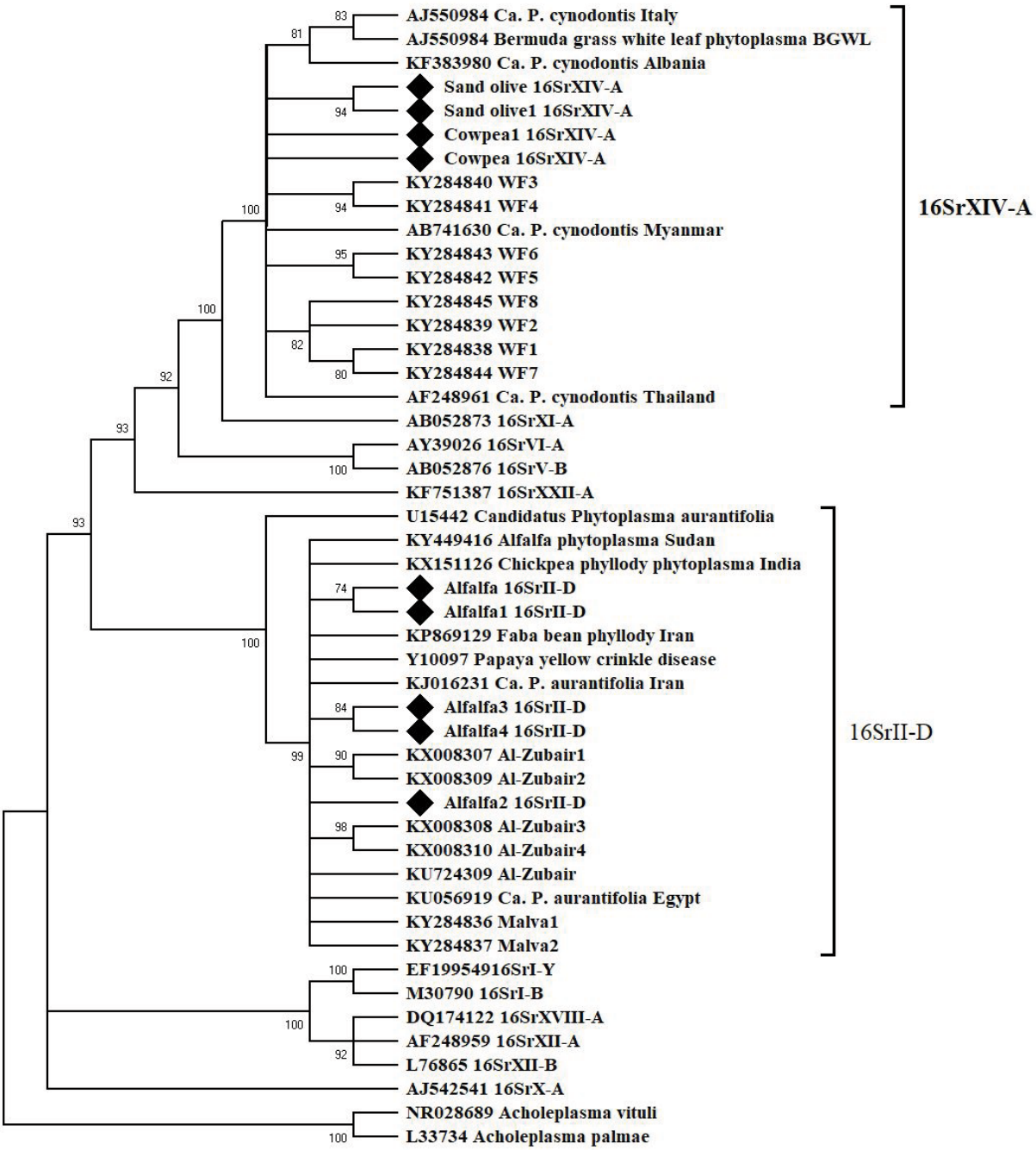

Fig. 2. Neighbor-joining phylogenetic tree constructed from partial 16S rDNA sequences (including 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence) of '*Ca*. Phytoplasma aurantifolia ' from alfalfa and '*Ca*. P. cynodontis' from sand olive and cowpea (marked with◆) and equivalent GenBank sequences from Iraq (GenBank accession numbers KU724309, KX008307-KX008310, KY284836-KY284837 and KY284838-KY284844) and different geographical locations. Phytoplasma sequences from different groups/subgroups were also included. *Acholeplasma vituli* (NR028689) and *A. palmae* (L33734) were included as an out-group comparison. The optimal tree with the sum of branch length = 0.59419532 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. This analysis involved 48 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1933 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*. 2018)

new plant hosts and diseases, namely sand olive witches broom and cowpea flat stem. This phytoplasma was reported to infect a wide range of hosts belonging to mono and dicot plants worldwide (Omar 2016; Rao *et al*. 2017b). When a suitable vector is present, these phytoplasmas can be a major problem for alfalfa, cowpea and sand olive and may limit their production in Iraq. Furthermore, poor flower quality produced by

symptomatic plants (i.e. phyllody) infected with phytoplasma may affect beekeeping in Iraq. Most Iraqi beekeepers grow alfalfa or disseminate bee hives in alfalfa fields to provide a source of food for the bees. The phytoplasma dissemination, through vectors or seeds, may threaten the environment since these phytoplasmas can be transmitted into Iraqi endogenous flora and fauna (mainly insect pollinators) (Gurr *et al.* 2015). The current study revealed the first association of '*Ca.* Phytoplasma cynodontis' and '*Ca.* Phytoplasma aurantifolia' with sand olive witches broom, cowpea flat stem and alfalfa little leaf diseases, respectively, in Iraq. Additional molecular survey studies are therefore required to investigate phytoplasma in other Iraqi provinces to provide information regarding the prevalence of phytoplasma in Iraq.

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