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DISEASE NOTES

First Report of 16Sr II ('*Candidatus* Phytoplasma aurantifolia') Subgroup-D Phytoplasma Associated with Alfalfa in Sudan

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In 2016, alfalfa (*Medicago sativa*) plants from four commercial fields in Sudan, each about 60 ha, were observed with leaf yellowing symptoms and stunted growth. Symptomatic plants were mostly concentrated around the edges of the fields with disease incidence of approximately 3 to 5%. Total nucleic acid was extracted from leaves of 14 symptomatic samples by a CTAB protocol. These extracts were used as the template in PCR assays with the universal phytoplasma primers P1/P7 (Smart et al. 1996). Eleven out of 14 samples produced an amplicon ~1.8 kb long. Subsequently, primer pairs R16F2n/R16R2 were used in a nested PCR using the P1/P7 PCR amplicon as a template (Gundersen and Lee 1996). Amplicons were obtained from the same 11 samples that tested positive with the first round of PCR, and no amplification was obtained by the nested PCR from the three samples that were negative with the P1/P7 primers. Four of the 11 products amplified using the P1/P7 primers were gel purified and cloned into the pGem T-Vector (Promega, Fitchburg, WI) according to the manufacturer's protocol. The consensus sequences were aligned using Geneious R9 (Biomaters, New Zealand). Three or four clones of each of the four positive samples (total of 14 clones) were sequenced in both directions. The percent identity among

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these 14 clones was 99.4 to 100%. BLASTn analysis revealed that the sequences obtained from the present study shared sequence similarity (99%) with the phytoplasma members 16SrII '*Candidatus* Phytoplasma aurantifolia': scaevola witches'-broom phytoplasma (AB257291) from Oman; eggplant big bud phytoplasma (JX441321) from Iran; 'Celosia argentea' phytoplasma (KX426374) from China; eggplant phyllody phytoplasma (FN257482) from Egypt; crotalaria witches'-broom phytoplasma (EU650181) from China; alfalfa witches'-broom phytoplasma (AB259169) from Oman; and mollicutes associated with papaya yellow crinkle disease (Y10097) from Australia. All of these are classified in the group 16SrII phytoplasmas using the *i*PhyClassifier virtual RFLP analysis tool. The complete sequences of the alfalfa phytoplasma strains (GenBank accession nos. KY449415–18) were subjected to a phylogenetic analysis. A phylogenetic tree was constructed by the neighbor-joining algorithm based on the 16S rRNA sequences. The results show that the Sudanese alfalfa isolates cluster with phytoplasmas in the 16SrII group. Virtual restriction fragment length polymorphism analyses of the 16S rRNA gene sequences were performed by using the *i*PhyClassifier online tool (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) to confirm the subgroup classification. The virtual pattern was identical to the reference pattern of 16Sr group II, subgroup D (Y10097). Several phytoplasma have been reported on alfalfa worldwide and the important groups are '*Ca*. P. fraxini'-16Sr VII-C from Argentina (Conci et al. 2005) and Stolbur phytoplasma-16SrXII-A and 16SrIII-B from Serbia (Starović et al. 2012). Previously, a phytoplasma from the 16SrII group was identified in diseased chickpea and faba bean from Sudan (Alfaro-Fernández et al. 2012). To the best of our knowledge, this is the first report of a phytoplasma, group 16SrII-D, infecting alfalfa in Sudan. The phytoplasma incidence in these fields warrants conducting surveys for this pathogen in other areas to study the epidemiology and impact of the disease on alfalfa production in Sudan and neighboring countries.

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