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First Report of '*Candidatus* phytoplasma solani' in Sunflower in Bulgaria

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The molecular identification of phytoplasma from infected sunflower in Bulgaria is reported. During a survey in the Boliarsko area in the Yambol region of Bulgaria, phytoplasma-like symptoms were observed on sunflower. The observed symptoms included yellowing, deformations and proliferation of flowers and sterile (empty) seeds. The Polymerase Chain Reaction (PCR) analyses with primers specific for the 16S ribosomal gene of phytoplasmas confirmed that symptomatic samples of sunflower were infected by phytoplasmas. Phytoplasmas were identified using sequence analyses of PCR amplified 16S rDNA. The obtained sequence showed identity with the '*Candidatus* Phytoplasma solani' strain from corn from Bulgaria and other 39 strains deposited in the GenBank. To our knowledge this is the first report of stolbur phytoplasma in sunflower (*Helianthus annuus*) in Bulgaria, adding a new cultivated plant species to the already wide natural host range of stolbur phytoplasma.

'*Candidatus* Phytoplasma solani' (stolbur phytoplasma-STOL) infects a wide range of cultivated and wild plants. Stolbur phytoplasma belongs to the 16SrXII-A ribosomal group and is transmitted by two planthoppers, *Hyalestes obsoletus* and *Reptalus panzeri* (Fos et al., 1992; Maixner, 1994; Jović et al., 2007; Quaglino et al., 2013). Considered as plant pathogen of European and Mediterranean origin, stolbur phytoplasma, also reported in other parts of the world, has been known in Bulgaria in tomato and pepper from 1970 (Kowachevski, 1971). Although known from before, stolbur phytoplasma was molecularly confirmed in Bulgaria in *Prunus avium,* grapevine (Bois noir) and in corn (corn reddening) in 2014 (Garnier, 2000; Duduk et al., 2010; Avramov et al., 2011; Mitrović et al., 2013; Genov et al., 2014). Until now, in sunflower, only phytoplasmas belonging to 16SrIII ribosomal group have been reported in Argentina (Guzmán et al., 2014).
Symptoms resembling those associated with phytoplasma diseases such as yellowing, deformations and proliferation of flowers and sterile (empty) seeds were observed in August 2012 in sunflower in the Boliarsko area, Bulgaria. Molecular analyses were performed to determine whether phytoplasmas are present in the symptomatic plants.

MATERIAL AND METHODS

Leaf samples were collected from three symptomatic and asymptomatic sunflower plants during August 2012, from Boliarsko location in the Yambol region of Bulgaria. Total nucleic acids were extracted from 0.5 g of fresh leaf midrib tissue from each sample, following the CTAB procedure described by Doyle and Doyle (1990), dissolved in TE buffer and stored at -20 °C. Nucleic acids were diluted in sterile distilled water 1:100 before performing PCR assays. For phytoplasma detection in collected samples, direct PCR assays with the universal phytoplasma primer pair P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) and nested PCR assays with primer pair

R16F2n/R2 (Lee et al., 1993; Gundersen and Lee 1996) were carried out. Each 25 µl PCR mix contained 1 µl of DNA template, 1× PCR Master Mix (Fermentas, Vilnius, Lithuania) and 0.4 µM of each primer. Samples lacking DNA were employed as negative controls. As a template for nested PCR, 1 µl of direct PCR amplicon diluted 30× in sterile water was used. Thirty-five PCR cycles were performed, for both amplifications under the following conditions: 1 min (2 min for the first cycle) for denaturation step at 94 °C, 2 min for annealing at 50 °C and 3 min (10 min for the last cycle) for primer extension at 72 °C. Six microliters of PCR products were separated on 1 % agarose gel, stained with ethidium bromide and visualized with a UV transilluminator.
The nested R16F2n/R2-amplified product of the selected symptomatic sample was purified using the mi-PCR purification kit (Metabion International AG, Martinsried, Germany). The product was sequenced by commercial service (Macrogen Inc., Seoul, South Korea) in both directions with the primers used for amplification (R16F2n/R2). The obtained sequences were assembled using Pregap4 from the Staden program package (Staden et al., 2000). The consensus sequence (1169 bp) is deposited in the NCBI, under the accession number KU556855, aligned with similar sequences of stolbur phytoplasmas publicly available in the GenBank using Clustal W (Thompson et al., 1997) from the Molecular Evolutionary Genetics Analysis program-MEGA6 (Tamura et al., 2013) and searched for SNPs in Bioedit program (Hall, 1999).

RESULTS AND DISCUSSION

Amplicons of the expected sizes (1.2 Kbp) were produced with DNA from two out of three symptomatic sunflower samples, while no amplification was observed with the DNA from asymptomatic plants and negative control. The sequence of selected strain obtained from R16F2n/R2 amplicon was 1169 bp in length, containing partial phytoplasma 16S rDNA.
The search for SNPs revealed no differences at any nucleotide position, showing identity (100 %) between the examined sunflower stolbur strain and the strain from corn from Bulgaria (KF907506), as well as with 39 other '*Ca*. Phytoplasma solani' strains. When compared with reference strain of '*Ca*. Phytoplasma solani' (AF248959) the Bulgarian sunflower strain sequence showed four nucleotide differences, making 99,66 % sequence homology.

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