Eur J Plant Pathol (2012) 133:791–795
DOI 10.1007/s10658-012-9975-7

Molecular identification of 16SrII-D subgroup phytoplasmas associated with chickpea and faba bean in Sudan

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Accepted: 14 March 2012 / Published online: 1 April 2012

Abstract In January 2011, symptomatic chickpea and faba bean plants were observed in fields located in the Gezira state (Sudan). Faba bean plants showed yellowing and stunting, whereas chickpea plants presented yellowing, reddening and little leaves. The disease etiology was investigated using nested polymerase chain reaction (PCR) with phytoplasma-specific primers which amplify a fragment of the 16S rRNA gene. Sequencing and restriction fragment length polymorphism (RFLP) analyses revealed that the tested phytoplasmas belonged to the group 16SrII. Phylogenetic analyses of the 16S rRNA gene of the obtained sequences indicated that the chickpea and faba bean phytoplasmas from Sudan were more closely related to the phytoplasmas subgroup 16SrII-D. To our knowledge, this is the first report of phytoplasmas from the group 16SrII-D infecting chickpea in Sudan, and faba bean worldwide.

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Keywords *Cicer arietinum* · Group 16SrII · PCR · RFLP · *Vicea faba*

Faba bean (*Vicea faba* L.) and chickpea (*Cicer arietinum* L.) are among the major food legumes cultivated in many countries worldwide and crop yield is often limited by disease problems. Phytoplasmas affect several hundred plant species, including vegetables, fruit crops, ornamental plants and trees in temperate to tropical regions (Schneider et al. 1997; Lee et al. 2000).

Phytoplasmas, formerly termed mycoplasma-like organisms, are cell-wall-less plant pathogenic bacteria belonging to the class Mollicutes. These organisms are restricted to the phloem tissues and, therefore, naturally transmitted by phloem-sap feeding insects, specifically leafhoppers (Families Cicadellidea), planthoppers (Families Fulgoridea) and psyllids (Families Psyllidea) (Lee et al. 2000). In general, plants infected by phytoplasmas exhibit symptoms that suggest profound disturbances in the normal balance of plant hormones or growth regulators, and include: virescence, phyllody, yellowing and reddening of leaves, witches' broom appearance, abnormal elongation of internodes, leaf rolling, general decline, bunchy appearance, stunting, floral abnormalities, shoot proliferation or reduced vigour (Lee et al.

2000; Bertaccini and Duduk 2009). Phytoplasmas were recently assigned to a novel genus *Candidatus* (*Ca.*) Phytoplasmas within the Mollicutes class, based on the percentage of similarity of the16S rRNA gene sequence (IRPCM 2004; Bertaccini and Duduk 2009). This study was undertaken to detect and identify the phytoplasmas present in the symptomatic samples of chickpea and faba bean plants collected in Sudan.

In January 2011, faba bean plants showing yellowing and general stunting and chickpea plants showing yellowing, reddening and little leaves were observed in fields of the Gezira state, Sudan. Samples of two and three chickpea and faba bean symptomatic plants were respectively collected. Four asymptomatic plants of both plant species were also included in the assay. Total DNA was extracted from the plant material using the cetyltrimethylammonium bromide (CTAB) buffer and the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) as described by Green and Thompson (1999). A nested-PCR was performed using the universal phytoplasma primers P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) in the first amplification, followed by R16F2n/R16R2 (Gundersen and Lee 1996) in the second amplification. Total DNA from the reference phytoplasmas of the different groups belonging to the Virology Group's (IAM-UPV) collection of isolates were used in this study as positive controls. These positive controls belonged to subgroups 16SrI-A (tomato big bud, BB), 16SrI-B (aster yellows, AY), 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. Fragments with the expected size of 1.2 kb were amplified by nested PCR only from the symptomatic faba bean and chickpea plants, and not from the asymptomatic plants or the water used as negative controls of the reaction.

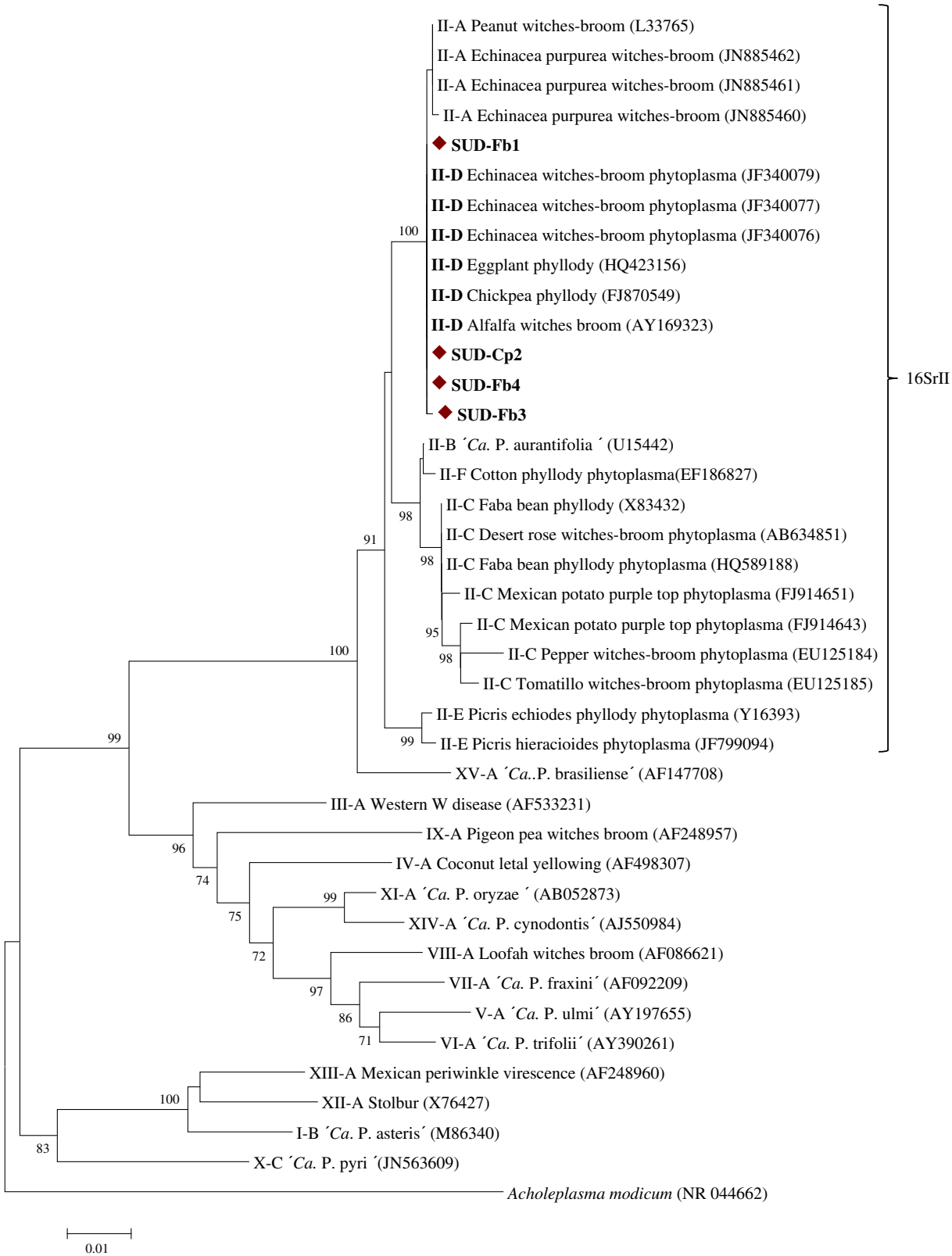
A restriction fragment length polymorphism (RFLP) analysis of the nested PCR products was performed to identify the specific phytoplasmas detected (Lee et al. 1998) with endonucleases *Alu*I, *Hae*III, *Hha*I, *Hpa*I *Kpn*I, *Mse*I, *Rsa*I and *Taq*I (MBI Fermentas, Vilnius, Lithuania). Restriction fragments were separated by electrophoresis through 5 % polyacrylamide (PAGE) gels, stained with ethidium bromide and visualised under UV illumination. The obtained patterns were compared with two DNA standard markers (GeneRuler™ 100 bp DNA Ladder Plus and pUC19DNA/MspI (*Hpa*II) marker, MBI Fermentas, Vilnius, Lithuania) and with the restriction profile of reference phytoplasmas of different groups. The comparison indicated that the chickpea and faba bean analyzed samples belonged to the group 16SrII.

To confirm the identity of the phytoplasmas detected, the 1.2 kb amplified products, were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and directly sequenced. The sequences obtained from faba bean (SUD-Fb1, SUD-Fb3, SUD-Fb4) and chickpea samples (SUD-Cp2) were deposited in the GenBank database under accession numbers JN233801, JN233803, JN233804 and JN233802, respectively. BLAST analyses revealed that these sequences shared an identity of more than 99.5 % among them and with different phytoplasma strains within the subgroup 16SrII-D as, for example, eggplant phyllody phytoplasma (accession number HQ423156). Similarity matrix, calculated using the Matrix Global Alignment Tool software, version 2.02 (http://bitincka.com/ledion/matgat) and performed with the different nucleotide sequences of phytoplasmas belonging to group 16SrII, also confirm those results (Table 1). Remarkably, sequences amplified with R16F2n/R16R2 primers of subgroups 16SrII-A and 16SrII-D presented similarities almost identical between them than within the subgroups. When analyzing the alignment of the sequences of strains belonging to subgroups II-A and II-D, mainly two nucleotide changes (positions 64 and 298 referred to sequence FJ870549) were observed between these subgroups. These changes correspond to the restriction site of endonucleases *Taq*I, *Mse*I and *Alu*I reported by some authors as RFLP differentiators of both subgroups (Khan et al. 2002; Lee et al. 1998). Sequences and RFLP analyses of the Sudanese phytoplasmas identified in this assay presented the same nucleotide changes and restriction profile as subgroup II-D. The rest of the subgroups of 16SrII, presented much more than two nucleotide changes.

A phylogenetic tree was constructed using the neighbour-joining method with MEGA (Molecular Evolutionary Genetics Analysis), version 5 (Tamura et al. 2011). The robustness of the inferred evolutionary relationships was assessed by 10,000 bootstrap pseudo-replicates. In this study, the phytoplasmas detected in Sudanese chickpea (SUD-Cp2) and faba bean (SUD-Fb1, SUD-Fb3 and SUD-Fb4) samples

Table 1 Analysis of the sequence similarities among the 16SrRNA gene sequences from the phytoplasmas grouped in the group 16SrII and the phytoplasmas identified in the chickpea and faba bean in Sudan. The similarity analysis was calculated using the Matrix Global Alignment Tool software (http://bitincka.com/ledion/matgat)

16SrRNA subgroup	Phytoplasma	Accession number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. II-A	Echinacea purpurea witches-broom	JN885461	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2. II-A	Peanut witches-broom	L33765	99.7	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3. II-B	*Ca.* P. aurantifolia	U15442	98.4	98.4	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4. II-C	Pepper witches-broom	EU125184	97.3	97.3	98.1	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5. II-C	Mexican potato purple top	FJ914651	98.1	98.1	98.8	98.4	100	–	–	–	–	–	–	–	–	–	–	–	–	–
6. II-C	Faba bean phyllody	HQ589188	98.4	98.4	99.1	98.7	99.5	100	–	–	–	–	–	–	–	–	–	–	–	–
7. II-C	Tomatillo witches-broom	EU125185	97.7	97.7	98.4	98.7	98.8	99.1	100	–	–	–	–	–	–	–	–	–	–	–
8. II-D	Echinacea witches-broom	JF340077	99.6	99.6	98.5	97.4	98.2	98.4	97.8	100	–	–	–	–	–	–	–	–	–	–
9. II-D	Eggplant phyllody	HQ423156	99.6	99.6	98.5	97.4	98.2	98.4	97.8	99.7	100	–	–	–	–	–	–	–	–	–
10. II-D	Chickpea phyllody	FJ870549	99.6	99.6	98.5	97.4	98.2	98.4	97.8	99.7	99.7	100	–	–	–	–	–	–	–	–
11. II-D	Alfalfa witches broom	AY169323	99.5	99.5	98.4	97.3	98.1	98.4	97.7	99.6	99.6	99.6	100	–	–	–	–	–	–	–
12. II-E	Picris echiodes phyllody	Y16393	98.2	98.2	98.2	97.1	97.8	98.1	97.4	98.3	98.3	98.3	98.2	100	–	–	–	–	–	–
13. II-E	Picris hieracioides	JF799094	98.2	98.2	98.0	96.9	97.6	97.9	97.3	98.3	98.3	98.3	98.2	99.4	100	–	–	–	–	–
14. II-F	Cotton phyllody	EF186827	98.4	98.4	99.4	98.3	99.0	99.3	98.6	98.5	98.5	98.5	98.4	98.2	98.0	100	–	–	–	–
15. SUD-Fb1	SUD-Fb1	JN233801	99.6	99.6	98.5	97.4	98.2	98.4	97.8	99.7	99.7	99.7	99.6	98.3	98.3	98.5	100	–	–	–
16. SUD-Cp2	SUD-Cp2	JN233802	99.6	99.6	98.5	97.4	98.2	98.4	97.8	99.7	99.7	99.7	99.6	98.3	98.3	98.5	99.7	100	–	–
17. SUD-Fb3	SUD-Fb3	JN233803	99.5	99.5	98.4	97.3	98.1	98.4	97.7	99.6	99.6	99.6	99.5	98.2	98.2	98.4	99.6	99.6	100	–
18. SUD-Fb4	SUD-Fb4	JN233804	99.6	99.6	98.5	97.4	98.2	98.4	97.8	99.7	99.7	99.7	99.6	98.3	98.3	98.5	99.7	99.7	99.6	100


II-A Peanut witches-broom (L33765)
II-A Echinacea purpurea witches-broom (JN885462)
II-A Echinacea purpurea witches-broom (JN885461)
II-A Echinacea purpurea witches-broom (JN885460)
SUD-Fb1
II-D Echinacea witches-broom phytoplasma (JF340079)
II-D Echinacea witches-broom phytoplasma (JF340077)
II-D Echinacea witches-broom phytoplasma (JF340076)
II-D Eggplant phyllody (HQ423156)
II-D Chickpea phyllody (FJ870549)
II-D Alfalfa witches broom (AY169323)
SUD-Cp2
SUD-Fb4
SUD-Fb3
16SrII
II-B ´Ca. P. aurantifolia ´ (U15442)
II-F Cotton phyllody phytoplasma(EF186827)
II-C Faba bean phyllody (X83432)
II-C Desert rose witches-broom phytoplasma (AB634851)
II-C Faba bean phyllody phytoplasma (HQ589188)
II-C Mexican potato purple top phytoplasma (FJ914651)
II-C Mexican potato purple top phytoplasma (FJ914643)
II-C Pepper witches-broom phytoplasma (EU125184)
II-C Tomatillo witches-broom phytoplasma (EU125185)
II-E Picris echiodes phyllody phytoplasma (Y16393)
II-E Picris hieracioides phytoplasma (JF799094)
XV-A ´Ca..P. brasiliense´ (AF147708)
III-A Western W disease (AF533231)
IX-A Pigeon pea witches broom (AF248957)
IV-A Coconut letal yellowing (AF498307)
XI-A ´Ca. P. oryzae ´ (AB052873)
XIV-A ´Ca. P. cynodontis´ (AJ550984)
VIII-A Loofah witches broom (AF086621)
VII-A ´Ca. P. fraxini´ (AF092209)
V-A ´Ca. P. ulmi´ (AY197655)
VI-A ´Ca. P. trifolii´ (AY390261)
XIII-A Mexican periwinkle virescence (AF248960)
XII-A Stolbur (X76427)
I-B ´Ca. P. asteris´ (M86340)
X-C ´Ca. P. pyri ´(JN563609)
Acholeplasma modicum (NR 044662)
100
98
91
98
95
98
100
99
99
96
74
99
75
72
97
86
71
100
83
0.01

◀ **Fig. 1** Phylogenetic tree of the 16S rRNA gene sequences constructed by the neighbour-joining method showing relationships among the phytoplasmas identified in the faba bean and chickpea plants from Sudan and the reference phytoplasmas retrieved from the GenBank database. The statistical reliability of the constructed trees was assessed by the bootstrap method based on 10,000 pseudoreplicates. The number above the nodes indicates the percentage of bootstrap replicates which supported branching. The scale bar represents a genetic distance of 0.01

were grouped in the clade of peanut witches'-broom strains (16SrII) described by White et al. (1998), specifically within the subgroup 16SrII-D (Fig. 1), together with Alfalfa witches'-broom (AY169323; Khan et al. 2002) or Chickpea phyllody phytoplasma (FJ870549; Akthar et al. 2008). The phylogenetic tree also demonstrated the close relationship between 16SrII-A and 16SrII-D.

Faba bean phyllody phytoplasma (FBP) was first described in Sudan, however it was later identified as 16SrII-C subgroup (Jones and Cockbain 1984; Lee et al. 1998; White et al. 1998; Bertaccini and Duduk 2009). By contrast, in this work the phytoplasma identified infecting faba bean belongs to the 16SrII-D subgroup. Therefore, this is the first report of a phytoplasma of these subgroup associated with faba bean plants. Within the group 16SrII or "peanut witches´-broom group", phytoplasmas of subgroup 16SrII-D have been recently identified in chickpea in Pakistan (Akthar et al. 2008). However, this is the first report of phytoplasmas of group 16SrII infecting chickpea in Sudan. More studies are needed to establish the incidence and impact of these phytoplasmas in Sudanese legume crops.

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