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MOLECULAR CHARACTERIZATION OF THE 16SR II GROUP OF PHYTOPLASMA ASSOCIATED WITH FABA BEAN (*Vicia faba* L.) IN SAUDI ARABIA

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ABSTRACT

This study was conducted to detect for the first time the occurrence of phytoplasma in symptomatic faba bean plants in Saudi Arabia. Faba bean is one of the most widely grown protein-producing food legumes. Ten leaf and stem samples showing symptoms of phyllody and stunting were collected from plants grown in Agricultural Research Station field Riyadh region, Saudi Arabia during December, 2011. The temperature in this area ranged between 15-20ºC. Two representative faba bean symptomatic leaves out of the total samples collected from the field in the visited area tested positive for plant pathogenic phytoplasmas using P1/P7 primer pair in the first round of PCR analysis. Nested PCR was conducted using the R16F2n/R16R2 primer pair, which yielded fragment of approximately 1.2 kb. No PCR products were obtained from the DNA extraction from asymptomatic plants. Phylogenetic analysis of the 16S rRNA gene of the obtained nucleotide sequence indicated that the two faba bean phytoplasmas isolates (faba bean phyllody and faba bean stunting) collected from Riyadh region of Saudi Arabia were more closely related to the phytoplasmas peanut witches'-broom group since its 16Sr RNA sequence showed a 97.2% to 99.3% identity with most members of this group. The nucleotide sequence for the Saudi Arabian isolates in this study were deposited in the GenBank with accession no. JQ861532 and JQ861533 respectively. The rest of the collected samples were assayed using cDNA probe for phytoplasma detection by dot and tissue blot hybridization. No hybridization was observed with DNA extracts from healthy plants.

Key words: Faba bean, Phytoplasma, PCR, 16SrII group, sequence, cDNA probe.

INTRODUCTION

Faba bean (*Vicia faba* L.) crop is one of the most extensively cultivated pulses in the Arabian region. The 2010 world production of faba beans was 4.3 million tons from 2.55 million hectares, which is relatively small compared with soybean and pea world production (262 and 10 million tons, respectively),(FAOSTAT, 2010). This crop was reported to be infected by many viruses, bacteria and fungal diseases in Saudi Arabia (Amira Hassan 2010), but not report yet to be infected by phytoplasmas disease agents. A member of the peanut witches'-broom phytoplasma group (16Sr II group) of the phytoplasma taxonomy formerly was known under the new invalidated species name 'Candidatus Phytoplasma australasiae' (Firrao *et al*. 2004). This phytoplasma aurantifolia group has been recorded from weeds, herbaceous and cultivated plants including fruits, vegetables, cereals and trees (McCoy *et al*. 1989; Tolu *et al*. 2006). Particularly in the Gulf region, the16Sr II group has been identified in lime (Zreik *et al*. 1995), alfalfa (Khan *et al*. 2002), sesame (Al-Sakeiti *et al*. 2005), alfalfa (Alhudaib 2009), lime (Alhudaib *et al*. 2009), and Tomato (Alhudaib and Rezk 2011). The 16Sr II group was detected in other crops in the Middle East region, including Iran and Lebanon (Weintraub and Jones 2010), Mediterranean region (Tolu *et al*. 2006), Australia (Aryamanesh *et al*. 2011), Mexico (Hernandez-Perez *et al*. 2009), Indonesia (Harling *et al*. 2009), Europe (Tolu *et al*. 2006; Davino *et al*. 2007; Parrella *et al*. 2008) and Sudan (Alfaro-Fernandez *et al*.2012; Zamora *et al*. 2012). More than 300 phytoplasma agents causing diseases in different plant species belonging to field crops, vegetables, trees, and weeds as well as in their insect vectors have been reported (Streten and Gibb 2006; Hoshi *et al.*, 2007; Parrella *et al*., 2008). In the 1990s, polymerase chain reaction (PCR) assays were developed for phytoplasma diagnostics (Deng and Hiruki 1991; Ahrens and Seemüller 1992; Lee *et al*. 1993; Schneider *et al*. 1995a; Gundersen and Lee 1996). The 16S rRNA gene is universal among prokaryotes and due to its conserved and variable regions; it provides a useful target for phytoplasma classification (Seemüller *et al*. 1998). All phytoplasma strains studied to date contain two rRNA operons (Firrao *et al*. 1996; Lauer and Seemüller 2000; Padovan *et al*. 2000; Marcone and Seemüller 2001) and a single tRNAIle in the spacer region between the 16S and 23S genes (Kuske and Kirkpatrick 1992; Smart *et al*. 1996). In the present study, we observed stunted plants

with shoe stringed leaves, phyllody and aborted flowers produced by phytoplasmas-like agents under tropical conditions. The aim of the present investigation was to detect and characterize, the agent associated with phytoplasma-like symptoms in faba bean fields for the first time in the Saudi Arabia.

MATERIALS AND METHODS

Source of the Faba bean phytoplasma samples: In December, 2011, ten leaves and stems samples of faba bean plants growing under tropical conditions showing typical phytoplasma-like symptoms which included stunting and phyllody (Fig.1) were collected from plants grown in Agricultural Research Station field, College of Food and Agriculture Sciences, King Saud University, Riyadh region, Saudi Arabia. Two of these samples were selected and analyzed for the presence of phytoplasmas using polymerase chain reaction (PCR) assays. These samples were chosen based on the symptoms expression, one sample show phyllody while the other one show stunting symptoms.

DNA extraction and PCR amplification: Total DNA was extracted from the symptomatic selected faba bean samples (leaf tissues) suspected with phytoplasma infection that were collected according to the protocol described by Arismendi *et al*. 2010. The DNA extracted from these samples was used as templates for the first round PCR using the universal phytoplasma primer pairs P1 and P7 (Deng and Hiruki 1991). For amplification, 1 µl DNA preparation from faba bean plants were used. Fifty microliters of PCR reaction mixture were added to each PCR tube containing the following reaction mixture 1.25 untis of the KAPA Long Range Hot Start DNA Polymerase, (2.5 u/µl, KAPA BIOSYSTEMS, BIOLINE, USA, Inc), 0.3 mM dNTPs (10 mM each dNTP), 1x of 5X KAPA Longe Range Buffer (without $Mgcl_2$), 1.75 mM $MgCl_2$ (25 mM), 10 µM of each primer P1/ P7 and PCR grade water up to 50 µl. The DNA amplification program was used according to Khan *et al.* 2002.

In nested PCR a 1:40 dilution of PCR products amplified by P1/P7 primer pair was used as template for a second round of PCR with primer pair R16F2n/R16R2 (Gundersen and Lee 1996). The mixture of the nested-PCR reaction (50 µl) was prepared as previously described for the first PCR. A total of 35 thermal cycles were carried out which included denaturation for 1 min (2 min for first cycle) at 94°C, annealing for 2 min at 50°C, and extension for 3 min (10 min in final cycle) at 72°C. Reactions were cycled in a Mastercycler (Eppendorf, Flexlid, Nexus gradient, Germany). For electrophoresis analysis, aliquots 10 µl each of PCR amplified DNA products were mixed with gel loading buffer. Separation was done on a 1% agarose gel in 1XTBE buffer (1x = 89 mM Tris, 89 mM borate, and 2 mM EDTA, pH 8.3). DNA was stained with ethidium bromide added to the gel at a concentration of 0.5 ug / ml. DNA was visualized on a UV transilluminator and photographed using DNA documentation gel analysis. DNA Hyper Ladder TM II ladder (BIOLINE, USA, Inc) was used to determine the size of RT- PCR amplified cDNA products.

DNA Sequencing and phylogenetic analyses: DNA fragment of the expected size from two Saudi Arabian isolate of phytoplasma affecting faba bean was excised and recovered with a Wizard PCR clean up kit (Promega, Madison, USA). The nucleotide sequences of these phytoplasma isolates were carried out in two directions with R16F2n/R16R2 primers at King Faisal Specialist Hospital & Research Center, Biological & Medical Research Department, Riyadh, Saudi Arabia using AB13730xl DNA Analyzer model HITACHI. Analyses of the obtained sequences were carried out using the DNAMAN software trial version 5.2.10 program (Lynnon BioSoft, Canada). To achieve a valid comparison, 22 phytoplasma isolate sequences obtained from GenBank database (table 1) were reduced to the longitude of the isolated sequences in this study.

Probe Generation, dot blot and tissue print hybridization: The cDNA probe was synthesized using PCR DIG Probe Synthesis Kit (Roche, Diagnostics GmbH, Mannheim, Germany) through PCR amplification from the phytoplasma infected faba bean plants. The rest of the collected samples were tested using cDNA probe for phytoplasma detection by dot blot hybridization. For dot blotting, sap extractions were prepared by grinding 50 mg of fresh plant tissues using the previous protocol for preparation of DNA as described by Arismendi *et al*. 2010. Five µl aliquots were spotted onto a nitrocellulose membrane. Stems were cut from different parts of each plant in the field and immediately printed on the nitrocellulose membranes. The membrane was denatured, air dried, irradiated with UV cross linker and kept at room temperature until hybridized according to El-Dougdoug *et al*. 2010. The prepared digoxigenin labeled cDNA probe was used in dot blot and tissue print hybridization assays. Pre-hybridization and hybridization were carried out at 68°C (under conditions of high stringency) essentially as described by Pallas *et al.* 1998. Binding reaction of the probe with the DNA samples was immunologically detected using 200 µl freshly prepared Nitro blue tetrazolume (NBT) and 5 -bromo-4-chloro-3-indolyl-phosphate(BCIB) stock solution (Roche, Diagnostics GmbH, Mannheim, Germany). The reaction was stopped by washing the membranes for 5 min with 50 ml of water. The results were documented by photocopying the wet filter by photography.

RESULTS

Disease symptoms and PCR amplification: A wide range of symptoms were remarked on naturally infected faba bean. The main symptoms were observed as chlorosis, proliferation and stunting (Fig. 1-A) and phyllody and reduction in leaves sizes (Fig. 1-B). Total DNA extracted from the two different symptomatic samples of faba bean was analyzed by PCR. With universal primer pair P1/P7, week DNA fragments of approximately 1,800 bp were amplified by first round PCR. While nested-PCR assay with the primer pair P1/P7 followed by the primer pair R16F2n/R16R2 yielded fragments of approximately 1.2 kbp.

Sequencing and phylogenetic analysis: To determine the 16Sr RNA group of the phytoplasma associated with faba bean stunting and phyllody, total DNA was extracted from the two symptomatic-bearing plants, and amplified by nested PCR using universal 16S rRNA phytoplasma primers R16F2n/R16R2. The nucleotide sequences were compared with the 16S rRNA gene sequences in the National Center for Biotechnology Information (NCBI) database. Phylogenetic analysis of the 16S rRNA gene of the obtained sequences indicated that the faba bean phylloy and stunting phytoplasma (FBP-Sa and FBS-Sa) isolates were more closely related (97.2-99.3%) to the phytoplasma peanut witches' -broom (16SrII) group since its 16SrDNA sequence showed a of identity with those of most of members of this group (Table 1and Figure 3). The two nucleotide sequences were deposited in NCBI accession no. JQ861532 and JQ861533 for faba bean phyllody and stunting phytoplasma respectively.

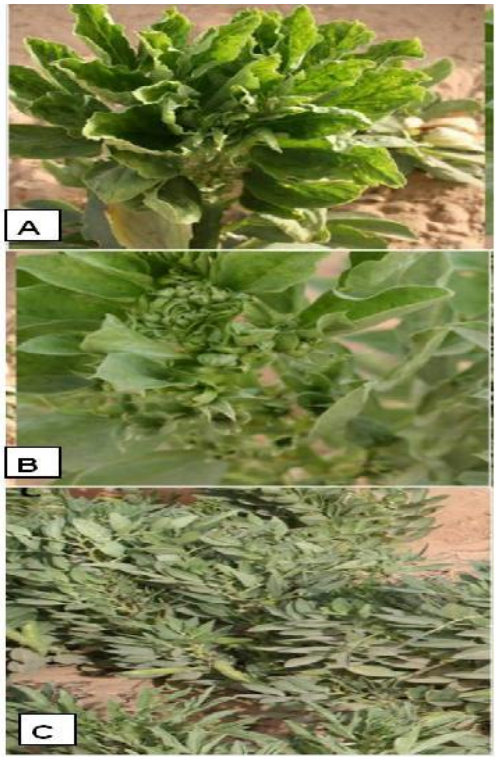

Fig. 1. Faba bean plants exhibiting (A): stunting and proliferation with shortened internodes (B): Phyllody and little leaves of buds symptoms at the base of the stem into shoots in infected faba bean plants and C: asymptomatic faba bean plant.

Dot blot and tissue printing hybridization assays: The digoxigenin-labeled probes specific for phytoplasma for 16S rDNA gene was used for tissue print (Fig. 4: A) and dot blot hybridization (Fig. 4: B). This probe gave positive purple color spots of hybridized nucleic acid extracted from the collected symptomatic plants (row a: FBP-Sa and row b: FBS-Sa) of faba bean plants from different parts under conditions of high stringency. The blots were showed strong hybridization signals in the area corresponding to the phloem in faba bean stem tissues when reacted with digoxgenin-labeled phytoplasma DNA probe. No signal was observed with DNA extraction from asymptomatic faba bean tissues (row c: 3, 4: Fig. 4). These results revealed the presence of phytoplasma infecting faba bean.

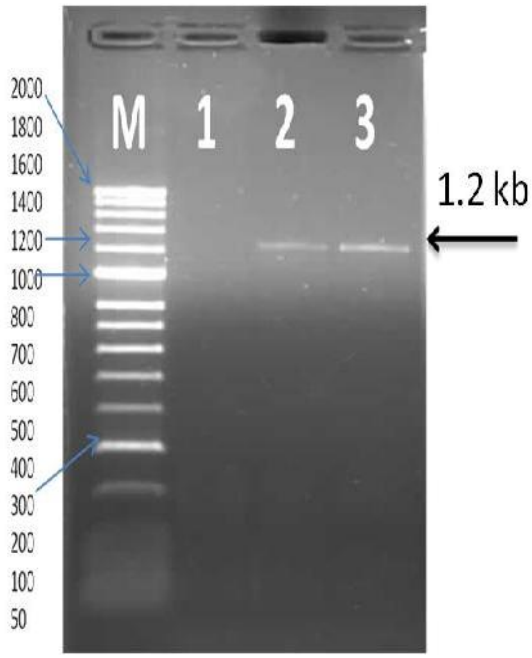

Fig. 2. Electrophoresis pattern of 16S rDNA fragment (1.2 kb) amplified by nested polymerase chain reaction from two symptomatic faba bean plants in Saudi Arabia (lanes 2-3). No PCR amplification from asymptomatic plant (negative control) (Lane 1); Lane M, DNA HyperLadder™ II ladder (BIOLINE).

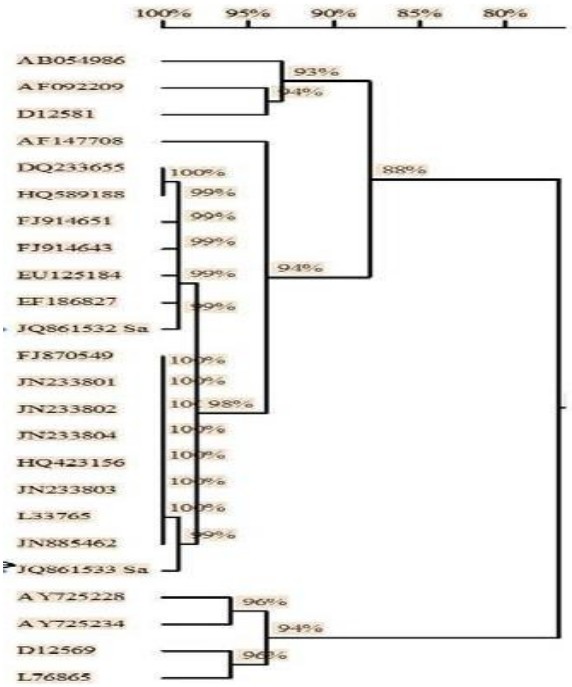

Figure 3: A Phylogenetic relationships among the faba bean phyllody and stunding phytoplasmas from Saudi Arabia and selected phytoplasmas referenced in NCBI including group 16SrII using DNAMAN software analysis

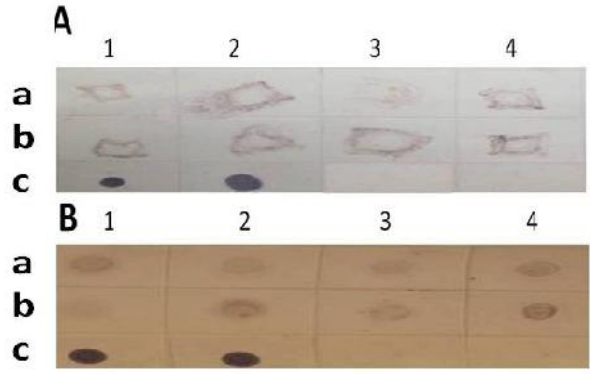

Fig. 4: A. Tissue blot hybridization using stem tissues (A) and dot blot hybridization using leaf tissues (B) from DNA of eight symptomatic faba bean. Phyllody (A: row: a & B: row: a) and stunting (A: row: b& B: row: b) on nitrocellulose membrane using dig-labeled DNA probe showing hybridization signals of phytoplasma with purple color concentrated on phloem tissues while no hybridization reaction with DNA extraction from asymptomatic plant (A and B: row c: 3, 4), DNA positive control (A and B: row c: 1, 2).

Table 1. Analysis of the sequence similarities among the 16S rRNA gene sequences from the phytoplasma grouped in the group 16SrII and the phytoplasma identified in the faba bean in Saudi Arabia. The similarity analysis was calculated using the DNAMAN Alignment software analysis.

Accession No.	Phytoplasma Name	group	JQ861532	JQ861533
AB054986	Candidatus Phytoplasma castaneae	16SrVI	86.2%	87.4%
AF092209	Candidatus Phytoplasma fraxini	16SrVII	86.5%	87.7%
AF147708	Candidatus Phytoplasma brasiliense	16SrXV	93.1%	93.8%
AY725228	Candidatus Phytoplasma graminis	16SrXII	82.4%	83.5%
AY725234	Candida Candidatus Phytoplasma caricae	16SrXII	82.1%	82.6%
L76865	Candidatus Phytoplasma australiense	16SrXII	83.8%	84.8%
D12581	Candidatus Phytoplasma oryzae	16SrXI	87.2%	88.5%
D12569	Onion yellows phytoplasma	16Sr I	83.6%	84.5%
EF186827	Cotton phyllody phytoplasma	16Sr II	98.9%	97.9%
EU125184	Pepper witches-broom phytoplasma	16Sr II	98.0%	97.2%
FJ870549	Chickpea phyllody	16Sr II	97.7%	99.2%
FJ914643	Mexican potato purple top phytoplasma	16Sr II	98.3%	97.4%
FJ914651	Mexican potato purple top phytoplasma	16Sr II	98.5%	97.7%
JN233801	'Vicia faba' stunting phytoplasma (SUDFb1)	16Sr II	98.1%	99.2%
JN233802	'Cicer arietinum' little leaf phytoplasma SUDCp2	16Sr II	98.1%	99.2%
JN233803	'Vicia faba' stunting phytoplasma SUDFb3	16Sr II	98.0%	99.3%
JN233804	'Vicia faba' stunting phytoplasma SUDFb4	16Sr II	98.1%	99.2%
JN885462	Echinacea purpurea witches-broom	16SrII	97.2%	99.1%
JQ861532	Saudi Arabia FB Phyllody	16Sr II	100%	97.3%
JQ861533	Saudi Arabia FB Stunting	16SrII	97.3%	100%
HQ423156	Eggplant phyllody phytoplasma	16Sr II	97.7%	99.2%
HQ589188	Faba bean phyllody phytoplasma	16Sr II	99.2%	97.9%
L33765	Peanut witches-broom	16SrII	97.6%	99.1%
DQ233655	Fars(Iran) alfalfa witches'-broom phytoplasma	16Sr II	99.1%	97.8%

DISCUSSION

Phytoplasma is considered to be one of the most important plant pathogens reducing the productivity of several economic crops. Symptoms due to phytoplasma infection are greatly variable according to the infected plants (Agrios 2005). There are different various methods available for demonstrating the presence of phytoplasma in diseased plants (Deeley *et al*. 1979; Hibben *et al*. 1986). Symptomatology is considered to be one of the major criteria for preliminary diagnosis of putative phytoplasma diseases. The symptoms observed in faba bean plants grown in the field did not resemble those produced by virus or fungal diseases and no phytoplasmas had been previously reported on faba bean in Saudi Arabia. Phytoplasmas have been reported to infect broad bean plants (Castro and Romero 2004; Schneider *et al*. 1995b). Phyllody symptoms of mung bean have been reported previously in India (Lakshmanan et al. 1988), Thailand (Chatchawankanphanich *et al*. 2000), and Australia (Wilson *et al*. 2001) and Pakistan (Akhtar *et al*. 2009). Moreira *et al*. 2010 reported that dry common bean plants (*Phaseolus vulgaris*) from the main production regions of Costa Rica have been affected by a disease. Main symptoms are a severe loss of bean pods due to flowering reduction or abortion, interveinal chlorosis, deformed leaves with corrugated midrib while diseased plants remaining green at harvest. Similar symptoms were previously associated with a phytoplasma infection in faba bean in Havana province and Sudan, therefore a possible phytoplasma infection was investigated (Alfaro-Fernandez *et al*. 2012; Zamora *et al*. 2012).

PCR assay not only provide a very sensitive means of detecting a specific phytoplasma strain but also, preferred in situations where the concentration of phytoplasma in a host is very low (Lee *et al*. 1994 , Al-Awadhi *et al*, 2002). PCR assays using universal or broad-spectrum primers designed based on conserved sequences allow the detection of a wide array of unknown phytoplasma associated with plants and insects by amplification of the 16S rDNA region (Lim and Sears 1989; Lee *et al*. 1994; Gundersen and Lee 1996 and Al-Awadhi *et al*. 2002). Amplifications and detection of 16S rDNA region using universal primer (R16mF2/R16mR1) resulted in a DNA size of approximately 1450 bp. Universal primer pair was employed successfully to detect a wide range of phytoplasma from plant sources (Lee *et al*. 1993). Nested-PCR assay, designed to increase both sensitivity and specificity of detection and amplification of phytoplasma from tested samples in which low titers are present, or substantial inhibitors are present that may interfere with the PCR efficacy (Lee *et al*. 1994; Gundersen and Lee 1996). PCR using a universal primer pair followed by a group-specific primer pair (nested-PCR) is capable of detection of dual or multiple phytoplasma present in the infected tissues in case of mixed infection (Lee *et al*. 1994). Nested- PCR with primer pair R16F2n/R16R2n resulted in DNA amplification of approximately 1200 bp. The use of nested PCR enables the detection of a secondary phytoplasma in cases of mixed infection and proved to be more specific than universal PCR (Al-Awadhi *et al*. 2002).

DNA Extracted from faba bean symptomatic and a symptomatic plant tissues (leaves and stems) were used for dot blot and tissue printing hybridization experiments to detect of this phytoplasma using nucleic acid hybridization assay. Our results showed that the DNA probe prepared during this study enabled the detection of this phytoplasma in faba bean plant. On the other hand, the DNA probe gave no signal with printing and extracts of healthy faba bean. In previously studies, DNA hybridization was used for identification of different phytoplasma associated with peanut, sweet potato witches'-broom and other phytoplasma disease (Chen and Lin 1997; Ko and Lin 1994). The only faba bean phytoplasma causing phyllody and stunting symptoms was described in the Sudan (Dafalla and Cousin 1988; Alfaro-Fernandez *et al*. 2012), and was classified by RFLPs in the 16SrII group (Saeed and Cousin 1995; Lee *et al*. 1998) or in the FBP group (Seemüller *et al*. 1998). This is thought to be the first report of a phytoplasma from the group 16SrII infecting faba bean in Sudan, but in Spain using sequence and Phylogenetic analysis indicated that this phytoplasma infecting Spainish faba bean clustered in the 16SrIII group (Castro and Romero 2004). This is thought to be the first report of a phytoplasma from this group infecting faba bean in Spain. Also the 16S rI group has been previously recorded in broad bean and sweet pepper and other vegetable crops in western Cuba (Arocha *et al*. 2007; Zamora *et al*.2012), and it has been also associated with 'amachamiento' disease of dry common bean in Costa Rica (Moreira *et al*. 2010). In our results, the Saudi Arabia faba bean phytoplasma was found to be taxonomically related to group16SrII, where the member type is peanut witches'-broom phytoplasma group based on sequence and phylogenetic analysis. This is the first report of a phytoplasma witches'-broom disease associated with faba bean phyllody and stunting infecting faba bean crop in Saudi Arabia and also the first of a Fabaceae phytoplasma in this country. More studies are needed to establish the incidence and impact of these phytoplasma other legume in Saudi Arabia.

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