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First Record of a Phytoplasma Associated with Faba Bean (*Vicia faba* L.) Witches'-broom in Egypt

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ABSTRACT

Symptoms of growth abnormalities and shoot proliferation followed by decline, whereas at the flowering stage virescence, phyllody and witches' broom prevailed naturally affecting faba bean plants were observed in fields of two governorate, Egypt. These plants were examined for phytoplasma infections. Light microscopy of hand-cut sections treated with Dienes' stain showed blue areas in the phloem region of phyllody-infected plants. Electron microscopy of ultrathin section of infected plants revealed phytoplasma units inside phloem tissues. Dodder was transmitted into healthy faba bean and periwinkle plants. DNA of the Phytoplasma was detected in symptomatic faba bean samples through the specific amplification of their 16S-23S rRNA gene using nested PCR. Universal phytoplasma-specific as well as witches' broom-specific PCR primers have been used. This is the first report of a phytoplasma infecting faba bean in Egypt.

Key words: Phytoplasma, witches' broom, nested PCR, DNA and faba bean (*Vicia faba*)

INTRODUCTION

Faba bean or broad bean (*Vicia faba* L.) is a major food and feed grain legume owing to the high nutritional value of its seeds which are rich in protein 27-34% (Link *et al*., 1995). In Egypt, faba bean is among the main nutritional source of plant proteins (Bakry *et al*., 2011). Its consumption exceeded 440,000 t in 2001 (FAO, 2002). Nevertheless, the total production of this crop is still limited and falls to cover the increasing local consumption, so there is a prerequisite to enlarge the production by expansion throughout reclaimed areas which signify the scope of cultivated lands (Khalafallah *et al*., 2008; Bakry *et al*., 2011).

According to the literature, the first report of phytoplasma from the group 16SrII-D in Sudan infecting faba bean worldwide was reported by Ana *et al*. (2012) and moreover, Castro and Romero (2004) was thought to be the first report of a phytoplasma infecting faba bean in Spain and the first report of a phytoplasma clustered in the 16SrIII group.

MATERIALS AND METHODS

Source of infected faba bean plants and symptomatology: Symptomatic plants showing phyllody disease (Fig. 1) and symptoms of growth abnormalities and shoot proliferation, whereas at the flowering stage virescence, phyllody and witches' broom prevailed naturally affecting

Fig. 1(a-f): Faba bean plants infected with phytoplasma: Symptoms of growth abnormalities and shoot proliferation (a and d) Witches' broom, (c and b) Phyllody, (e) Flowering stage virescence and (f) compared to the healthy ones

Faba bean cv. Giza 3 and 40 plants were observed in fields of two governorate, Egypt. Samples of infected plants were collected from two locations at Al-Wadi Al-Gaded and one at Qena governorate. grown infected plants were laced root in plastic bag with a moist towel.

Dienes' stain by light microscopy: Plant samples are taken from shoots and petioles leaves of infected and healthy control plants Free hand sections were prepared by hand and transferred into distilled water, using razor blade edge. The sections were transferred to Dienes' stain for 10 min. The stain was prepared by dissolving 2.5 g methylene blue, 1.25 g azure 11, 10.0 g maltose and 0.25 g sodium carbonate (Na_2CO_3) in 100 mL distilled water filter through filter paper and dilute to 0.2% (v/v) in distilled water according to Deeley *et al.* (1979), Hibben *et al.* (1986) and Musetti (2013). The stained sections were later washed in distilled water and mounted in distilled water on a glass slide and examined microscopy.

Dodder transmission: The transmission experiments were carried out to transmit phytoplasma from the symptomatic phyllody-faba bean plants to periwinkle and healthy faba bean by dodder (*Cucusta campestris*) transmission. plants were kept in the insect-proof greenhouse. In the case of dodder transmission, dodders was grown from seeds dodder *in vitro* germinated on Petri dishes 12 cm diameter bottomed with wetted filter paper for 4 days at room temperature (27-30°C) and placed on the recipient host, periwinkle. The symptomatic Faba beans were used as donor plants and dodders were used as the bridge between the recipient host and donor plants. The dodder bridges were left for 3-4 weeks for transmission before discarded. The symptom development on periwinkle and healthy faba bean was recorded.

Detection by electron microscopy of ultra thin section of phytoplasma: Tissues of infected faba bean petioles and main veins were cut into small pieces about 1-2 mm., fixed in 2% glutraldhyde in 0.1 M Na-Cacodylate buffer, pH 7.2 and subjected to a vacuum for 1-4 min every 15 min for 2 h on ice. Prior to vacuum treatment, floating samples were poked under the buffer surface with pointed metal pokers. Rinsing took place in 0.1 M Na-Cacodylate buffer, pH 7.2, for 45 min, with buffer changes at 15 and 30 min. Further fixation in 1% Osmium Tetraoxide in Na-Cacodylate buffer, under intermittent vacuum and poking, took place for 1.5 h. Samples were then rinsed again in the Na-Cacodylate buffer. Dehydrated Samples were dehydrated through an Ethanol series in buffer: 35-50-70-80-95-100-100% for 60 min each. Then Infiltrate with res in Semi thin sections were prepared on glass slides through cutting at 1 μm using the ultramicrotome. Sections were stained with Toludine blue for 5 min and examined by light microscope model M-200 M.

Ultra-thin sections were cut using ultramicrotome Leica model EM-UC6 at thickness 90 nm, mounted on copper grids (400 mish). Sections were stained with double stain (Uranyl acetate 2% 10 min followed by Lead citrate for 5 min and examined by transmission electron microscope JEOL (JEM-1400) at the candidate magnification. Images were captured by CCD camera model AMT, optronics camera with 1632×1632 pixel formate as side mount configuration (Osmont and Freeling, 2001).

Molecular diagnosis: DNA was extracted from Faba bean tissues showing Witches'-Broom's symptoms as well as healthy faba bean plants using column DNA extraction method (Sigma, USA) according to the manufacture manual. Both naturally and experimentally infected Faba bean plants were used.

The total DNA extracted from healthy and infected plants was used individually as template for PCR. The DNA of the Phytoplasma was detected in symptomatic faba bean samples through the specific amplification of their 16S-23S rRNA gene by PCR using the universal phytoplasma primer pair P1/P7 in direct PCR followed by primer pair R16F2n/R16R2 in nested PCR (Lee *et al.*, 2004; Sinclair *et al.*, 2000; Bhat *et al.*, 2006). The PCR mixture contained 25 pmol of each primer; 20 ng of template DNA; 200 μM of each dNTP; 1×polymerase reaction buffer; 2.5 mM MgCl2; 1.25 U of dream-Taq polymerase (Fermentas) and sterile water to a final volume of 25 μL. Amplification was started with denaturation at 94°C for 3 min followed by 35 cycles starting with denaturation at 94°C for 1 min, annealing for 2 min at 55°C and primer extension for 3 min at 72°C with a final extension step at 72°C for 10 min. One microlitre of (1:10) diluted PCR products from the first amplification was used as template in the second round PCR (nested PCR) with the same PCR parameters and reaction conditions. Faba bean witches' broom phytoplasma was detected through

direct PCR using witches' broom-specific primers SR1: 5'-agg cgg atc ctt ggg gtt aag tcg taa-3 and SR2: 5'-agg cga att ccg tcc ttc atc ggc tct t-3' representing the phytoplasma-specific 16S/23S rRNA (rDNA) intergenic Spacer Region (SR) (Liefting *et al.*, 1996; Smart *et al.*, 1996). Amplification was started with a denaturation step at 94°C for 2 min followed by 5 cycles at 94°C for 15 sec, 45°C for 15 sec and 72°C for 30 sec followed by 25 cycles of 15 sec at 94°C, 15 sec at 55°C and 30 sec at 72°C, with a final extension of 10 min at 72°C. The PCR products were stained with gel star (Lonza, USA) and analyzed by electrophoresis in 1.0% agarose gel and visualized by UV illumination using Gel Documentation System (Gel Doc 2000, Bio-Rad, USA).

RESULTS

Source of plant and symptomatology: Generally, in the most of fields, symptoms of growth abnormalities, phyllody disease and yellowing of leaves appeared on Faba bean cv. Giza 3 and 40 plants (Fig. 1a). Moreover, symptoms of shoot proliferation, whereas at the flowering stage virescence, phyllody and witches' broom prevailed were observed also (Fig. 1b-e) compared to the healthy ones (Fig. 1f).

Dienes' stain by Light microscopy Faba bean sample sections of leaf petiole from symptomatic leaves, stained with Dienes, showed some phloem cells are stained in blue, thus positive leaf petiole (Fig. 2b) in comparison with healthy ones (Fig. 2a).

Dodder transmission: The transmission experiments by dodder transmission were carried out to transmit phytoplasma from the phyllody faba bean plants to periwinkle and healthy faba bean transmission. The phytoplasma that causes phyllody disease was successfully transmitted from infected to healthy plants. The causative agent was successfully transmitted to healthy plants, producing disease Symptoms within 25-35 days.

Detection by electron microscopy of ultra thin section of phytoplasma: Ultrathin sections of petiole leaves and shoot tissues showed phytoplasma-like organisms localized at the periphery of a parenchyma phloem cell almost completely units were rounded, elongated, measuring 200-400 nm in size, bounded by a unit membrane (Fig. 3).

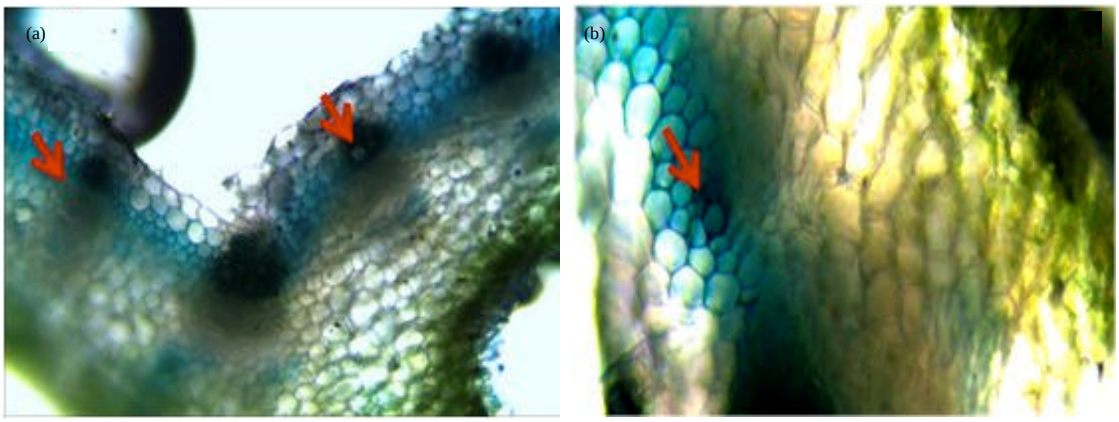

Fig. 2(a-b): Dienes' stain by light microscopy showed some phloem cells are stained in blue refers to (a) Positive leaf petiole and (b) Compared with healthy ones

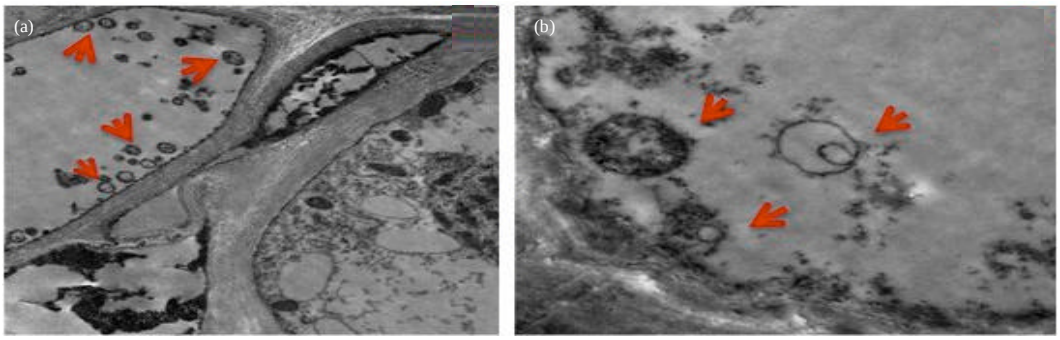

Fig. 3(a-b): Electron microscopic examinations of ultrathin sections of (a) Petiole leaves and (b) Shoot tissues. Completely units were rounded, elongated, measuring 200-400 nm in size, bounded by a unit membrane

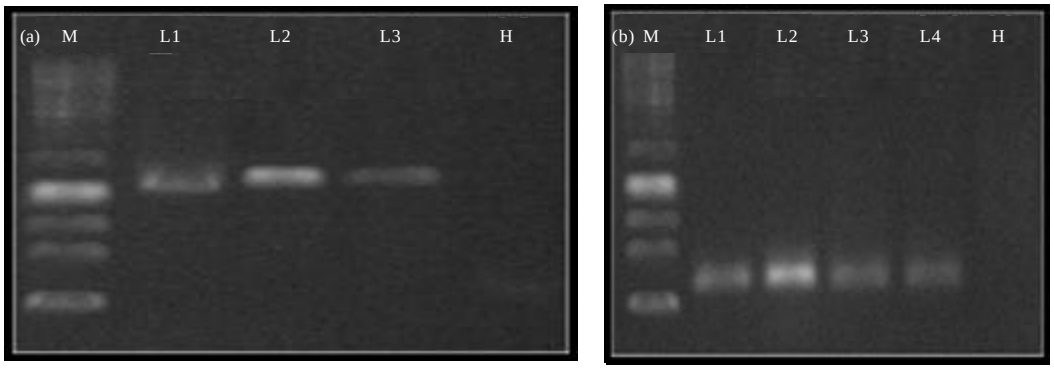

Fig. 4(a-b): (a) Gel electrophoresis for the detection of the phytoplasma in faba bean symptomatic leaves using universal phytoplasma-specific PCR primers and (b) Witches' broom-specific PCR primers. M: 1 Kb DNA ladder, L1 and L2: Naturally infected and L3, L4: Experimentally infected samples showed W. B symptoms. H: Healthy plant control

Molecular detection of phytoplasma: Total DNA isolated from infected faba bean plants was used for the PCR detection of phytoplasma. All symptomatic samples, naturally and/or experimentally infected with phytoplasma but not healthy plants showed individually clear band at ~1200 bp as a result for the nested PCR amplification utilizing the universal phytoplasma-specific primer R16F2n/R16R2 (Fig. 4a). Direct PCR using witches' broom-specific primers SR1/SR2 was used to detect Faba bean witches' broom phytoplasma in all symptomatic plants. Electrophoresis analysis of the PCR product showed a single amplified fragment of ~325 bp while no fragments were amplified from the DNA extracted from symptomless or healthy plants (Fig. 4b).

DISCUSSION

Phytoplasma occurred worldwide and associated with several hundred diseases affecting economically important corps in the most of fields.

In this report, phytoplasma affected faba bean plants in fields of two governorate, Egypt and symptoms of growth abnormalities and shoot proliferation followed by decline, whereas at the flowering stage virescence, phyllody and witches' broom were observed. these results were in agree with (Dafalla and Cousin, 1988) who observed phyllody of faba bean plants in fields located in the Gezira state (Sudan) and the disease was characterized by a series of floral proliferation including virescence, phyllody and proliferation of sprouts together with other growth abnormalities, such as loss of apical dominance, vivipary and enhanced vegetative growth. moreover, Castro and Romero (2004) was thought to be the first report of a phytoplasma infecting faba bean in Spain, showing symptoms of shoe stringed leaves, phyllody and flower abortion were observed in fields of Antequera.

Dodder transmission were carried out to transmit phytoplasma from the phyllody faba bean plants to periwinkle and healthy faba bean transmission; also, Dafalla and Cousin (1988) observed phyllody of faba bean plants in fields located in the Gezir a state (Sudan).

The disease agent was also transmitted through dodder to the differential host *Catharanthus roseus* (El-banna *et al.*, 2007).

Transmission of phytoplasmas from naturally infected plant host species using the parasitic plant *Cuscuta* spp. (dodder) to *Catharanthus roseus* (Madagascar periwinkle) is an effective way to maintain a wide range of phytoplasmas for further research. Here, we describe transmission via dodder from an infected medicinal plant *Rehmannia glutinosa* var. purpurea and from a symptomatic redcurrant plant (*Ribes* spp.) to *C. roseus* indicator plants using a "stable bridges" method. In both cases, typical symptoms of phytoplasma disease on periwinkle plants were obtained: Virescent flowers with an increased number of axillary shoots and smaller leaves after transmission from *R. glutinosa* and greening petals (virescence) after transmission from *Ribes* spp. (Pribylova and Spak, 2013).

Many authors have used electron microscopy to reveal the presence of Phytoplasma in the phloem tissues and to study cytological interactions between these pathogens and their hosts.

Light microscopy techniques have been used successfully as preliminary methods for diagnosis to verity the presence of phytopalsma in symptomatic plants so they constitute the first steps towards understanding the possible association between phytoplasma and the disease symptoms in the plants. Moreover, LM method is fast and less expensive than electron microscopy techniques.

Molecular diagnosis confirmed the infection of faba bean (*Vicia faba* L.) with Phytoplasma Associated with Witches'-Broom for the first time in Egypt. The DNA extracted from the faba bean plants, those showed phyllody and/or witches' broom symptoms, was used as template for nested PCR utilizing universal phytoplasma-specific PCR primers and witches' broom-specific PCR primers, respectively. The results of the nested PCR using the universal phytoplasma-specific primers showed a clear band at the specific size 1200 bp while the direct PCR using the witches' broom-specific PCR primers showed a clear band at ~325 bp. Those PCR results clearly demonstrated the natural infection of faba bean with Phytoplasma Associated with Witches'-Broom. PCR results for faba bean and periwinkle plants those were experimentally infected with phytoplasma confirmed the successful transmission of the Phytoplasma Associated with Witches'-Broom into the healthy faba bean and periwinkle plants using Dodders.

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